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(54) Title: NOVEL HUMAN KALLIKREIN-LIKE GENES

(57) Abstract

The invention relates to nucleic acid molecules, kallikrein-like proteins encoded by such nucleic acid molecules; and use of the proteins and nucleic acid molecules

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TITLE: Novel Human Kallikrein-Like Genes

FIELD OF THE INVENTION

The invention relates to nucleic acid molecules, proteins encoded by such nucleic acid molecules; and use of the proteins and nucleic acid molecules

BACKGROUND OF THE INVENTION

Kallikreins and kallikrein-like proteins are a subgroup of the serine protease enzyme family and exhibit a high degree of substrate specificity (1). The biological role of these kallikreins is the selective cleavage of specific polypeptide precursors (substrates) to release peptides with potent biological activity (2). In mouse and rat, kallikreins are encoded by large multigene families. In the mouse genome, at least 24 genes have been identified (3). Expression of 11 of these genes has been confirmed; the rest are presumed to be pseudogenes (4). A similar family of 15-20 kallikreins has been found in the rat genome (5) where at least 4 of these are known to be expressed (6).

Three human kallikrein genes have been described, i.e. prostatic specific antigen (PSA or KLK3) (7), human glandular kallikrein (KLK2) (8) and tissue (pancreatic-renal) kallikrein (KLK1) (9). The PSA gene spans 5.8 Kb of sequence which has been published (7); the KLK2 gene has a size of 5.2 Kb and its complete structure has also been elucidated (8). The KLK1 gene is approximately 4.5 Kb long and the exon sequences and the exon/intron junctions of this gene have been determined (9).

The mouse kallikrein genes are clustered in groups of up to 11 genes on chromosome 7 and the distance between the genes in the various clusters can be as small as 3-7 Kb (3). All three human kallikrein genes have been assigned to chromosome 19q13.2 – 19q13.4 and the distance between PSA and KLK2 has been estimated to be 12 Kb (9).

A major difference between mouse and human kallikreins is that two of the human kallikreins (KLK2 and KLK3) are expressed almost exclusively in the prostate while in animals none of the kallikreins is localized in this organ. Other candidate new members of the human kallikrein gene family include protease M (10) (also named Zyme (11) or neurosin (12) and the normal epithelial cell-specific gene-1 (NES1) (13). Both genes have been assigned to chromosome 19q13.3 (10,14) and show structural homology with other serine proteases and the kallikrein gene family (10-14).

SUMMARY OF THE INVENTION

In efforts to precisely define the relative genomic location of PSA, KLK2, Zyme and NES1 genes, an area spanning approximately 300 Kb of contiguous sequence on human chromosome 19 (19q13.3 –q13.4) was examined. The present inventors were able to identify the relative location of the known kallikrein genes and, in addition, they identified other kallikrein- like genes which exhibit both location proximity and structural similarity with the known members of the human kallikrein family. The novel genes exhibit homology with the currently known members of the kallikrein family and they are co-localized in the same genomic region. These new genes, like the already known kallikreins have utility in various cancers including those of the breast, testicular, and prostate.

The kallikrein-like proteins described herein are individually referred to as "KLK-L1, KLK-L2,

KLK-L3, KLK-L4, KLK-L5, or KLK-L6", and collectively as "kallikrein-like proteins" or "KLK-L Proteins". The genes encoding the proteins are referred to as " *klk-l1, klk-l2, klk-l3, klk-l4, klk-l5, or klk-l6*", and collectively as "kallikrein-like genes" or "*klk-l* genes".

Broadly stated the present invention relates to an isolated nucleic acid molecule which comprises:

(i) a nucleic acid sequence encoding a protein having substantial sequence identity with an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;

(ii) a nucleic acid sequence encoding a protein comprising an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;

(iii) nucleic acid sequences complementary to (i);

(iv) a degenerate form of a nucleic acid sequence of (i);

(v) a nucleic acid sequence capable of hybridizing under stringent conditions to a nucleic acid sequence in (i), (ii) or (iii);

(vi) a nucleic acid sequence encoding a truncation, an analog, an allelic or species variation of a protein comprising an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively; or

(vii) a fragment, or allelic or species variation of (i), (ii) or (iii).

Preferably, a purified and isolated nucleic acid molecule of the invention comprises:

(i) a nucleic acid sequence comprising the sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65 wherein T can also be U;

(ii) nucleic acid sequences complementary to (i), preferably complementary to the full nucleic acid sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65;

(iii) a nucleic acid capable of hybridizing under stringent conditions to a nucleic acid of (i) or (ii) and preferably having at least 18 nucleotides; or

(iv) a nucleic acid molecule differing from any of the nucleic acids of (i) to (iii) in codon sequences due to the degeneracy of the genetic code.

The invention also contemplates a nucleic acid molecule comprising a sequence encoding a truncation of a KLK-L protein, an analog, or a homolog of a KLK-L Protein or a truncation thereof. (KLK-L Proteins and truncations, analogs and homologs of KLK-L Proteins are also collectively referred to herein as "KLK-L Related Proteins").

The nucleic acid molecules of the invention may be inserted into an appropriate expression vector, i.e. a vector that contains the necessary elements for the transcription and translation of the inserted coding sequence. Accordingly, recombinant expression vectors adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements linked to the nucleic acid molecule.

The recombinant expression vector can be used to prepare transformed host cells expressing KLK-

L Related Proteins. Therefore, the invention further provides host cells containing a recombinant molecule of the invention. The invention also contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule comprising a nucleic acid molecule of the invention, in particular one which encodes an analog of a KLK-L Protein, or a truncation of a KLK-L Protein.

The invention further provides a method for preparing KLK-L Related Proteins utilizing the purified and isolated nucleic acid molecules of the invention. In an embodiment a method for preparing a KLK-L Related Protein is provided comprising (a) transferring a recombinant expression vector of the invention into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of the KLK-L Related Protein; and (d) isolating the KLK-L Related Protein.

The invention further broadly contemplates an isolated KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.

The KLK-L Related Proteins of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins.

The invention further contemplates antibodies having specificity against an epitope of a KLK-L Related Protein of the invention. Antibodies may be labeled with a detectable substance and used to detect proteins of the invention in tissues and cells.

The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and/or to proteins of the invention. Therefore, the invention also relates to a probe comprising a nucleic acid sequence of the invention, or a nucleic acid sequence encoding a protein of the invention, or a part thereof. The probe may be labeled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleic acid molecule of the invention including nucleic acid molecules coding for a protein which displays one or more of the properties of a protein of the invention.

The invention still further provides a method for identifying a substance which binds to a protein of the invention comprising reacting the protein with at least one substance which potentially can bind with the protein, under conditions which permit the formation of complexes between the substance and protein and detecting binding. Binding may be detected by assaying for complexes, for free substance, or for non-complexed protein. The invention also contemplates methods for identifying substances that bind to other intracellular proteins that interact with a KLK-L Related Protein. Methods can also be utilized which identify compounds which bind to KLK-L gene regulatory sequences (e.g. promoter sequences).

Still further the invention provides a method for evaluating a compound for its ability to modulate the biological activity of a KLK-L Related Protein of the invention. For example a substance which inhibits or enhances the interaction of the protein and a substance which binds to the protein may be evaluated. In an embodiment, the method comprises providing a known concentration of a KLK-L Related Protein, with a substance which binds to the protein and a test compound under conditions which permit the formation of complexes between the substance and protein, and removing and/or detecting complexes.

Compounds which modulate the biological activity of a protein of the invention may also be identified using the methods of the invention by comparing the pattern and level of expression of the protein of the invention in tissues and cells, in the presence, and in the absence of the compounds.

The proteins of the invention and substances and compounds identified using the methods of the invention, and peptides of the invention may be used to modulate the biological activity of a KLK-L Related Protein of the invention, and they may be used in the treatment of conditions such as cancer (e.g. breast, testicular, and prostate cancer). Accordingly, the substances and compounds may be formulated into compositions for administration to individuals suffering from cancer.

Therefore, the present invention also relates to a composition comprising one or more of a protein of the invention, a peptide of the invention, or a substance or compound identified using the methods of the invention, and a pharmaceutically acceptable carrier, excipient or diluent. A method for treating or preventing cancer is also provided comprising administering to a patient in need thereof, a KLK-L Related Protein of the invention, or a composition of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows an approximate 300 Kb of contiguous genomic sequence around chromosome 19q13.3 - q13.4 represented by 8 contigs, each one shown with its length in Kb. The contig numbers refer to those reported in the Lawrence Livermore National Laboratory website. Note the localization of the seven known genes (PSA, KLK2, Zyme, NES1, HSCCE, neuropsin and TLSP) (see abbreviations for full names of these genes). All genes are represented with arrows denoting the direction of transcription. The gene with no homology to human kallikreins is termed UG (unknown gene). The five new kallikrein-like genes (KLK-L1 to KLK-L5) were numbered from the most centromeric to the most telomeric. Numbers just below or just above the arrows indicate appropriate Kb lengths in each contig. Gene lengths and distances between genes are rounded to the nearest 6.5 kb. The site of the gap is marked with an asterisk.

Figure 2 shows a contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Distances between genes are in base pairs.

Figure 3 shows tissue expression of the prostase/KLK-L1 gene as determined by RT-PCR. Actin and PSA are control genes. Interpretations are presented in Table 9.

Figure 4 shows the sequence of PCR product obtained with cDNA from female breast tissue using prostase/KLK-L1 primers. Primer sequences are underlined. The sequence is identical to the sequence obtained from prostatic tissue.

Figure 5 is a blot showing the results of experiments for hormonal regulation of the prostase/KLK-

L1 gene in the BT-474 breast carcinoma cell lines. DHT = dihydrotestosterone. Steroids were added at 10^{-8} M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (up-regulated by androgens and progestins), are control genes. Prostase/KLK-L1 is up-regulated by androgens and progestins.

Figure 6 is a schematic diagram showing comparison of the genomic structure of PSA, KLK1, KLK2, zyme, neuropsin and prostase/KLK-L1 genes. Exons are shown by open boxes and introns by the connecting lines. Arrow head shows the start codons and the vertical arrow represents stop codons. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs.

Figure 7 shows the genomic organization and partial genomic sequence of the KLK-L2 gene. Intronic sequences are not shown except for the splice junctions. Introns are shown with lower case letters and exons with capital letters. The start and stop codons are encircled and the exon –intron junctions are boxed. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are inside triangles. Putative polyadenylation signal is underlined.

Figure 8 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4.Genes are represented by horizontal arrows denoting the direction of the coding sequence. Distances between genes are mentioned in base pairs .

Figure 9 shows the alignment of the deduced amino acid sequence of KLK-L2 with members of the kallikrein multi-gene family. Genes are (from top to bottom) : Prostase/KLK-L1, enamel matrix serine proteinase 1 (EMSP1) (GenBank accession # NP_004908), KLK-L2, zyme (GenBank accession # Q92876), neuropsin (GenBank accession # BAA28673), trypsin-like serine protease (TLSP) (GenBank accession # BAA33404), PSA (GenBank accession # P07288), KLK2 (GenBank accession # P20151), KLK1 (GenBank accession # NP_002248), and trypsinogen (GenBank accession # P07477). (See SEQ.ID. NOs. 68-77) Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are represented by (✤) and the 29 invariant serine protease residues by (▮ or ✤). Conserved areas around the catalytic triad are boxed. The predicted cleavage sites are indicated by (♠). The dotted area represents the kallikrein loop sequence. The trypsin like cleavage pattern is indicated by (✪).

Figure 10(A) shows a dendrogram of the predicted phylogenetic tree for some kallikrein genes. Neighbor-joining/UPGMA method was used to align KLK-L2 with other members of the kallikrein gene family. Gene names and accession numbers are listed in Figure 9. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L2 gene in one group with EMSP, prostase, and TLSP. (B) Plot of hydrophobicity and hydrophilicity of KLK-L2.

Figure 11 is a blot showing tissue expression of KLK-L2 gene as determined by RT-PCR. Actin and PSA are control genes. Interpretations are presented in Table 12.

Figure 12 is a blot showing hormonal regulation of the KLK-L2 gene in BT-474 breast carcinoma

cell lines. DHT = dihydrotestosterone. Steroids were at 10^{-8} M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins), are control genes. KLK-L2 is upregulated by estrogens and progestins.

Figure 13 are blots of EtBr-stained agarose gels. Total RNA was extracted from normal, benign, and cancer tissues and used to generate cDNA. PCR was performed on cDNA

Figure 14 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4.Genes are represented by horizontal arrows denoting the direction of the coding sequence. Gene lengths and distances between genes are rounded to the nearest 0.5 kb. The site of the gap is marked with an asterisk. Telomeric to TLSP there are likely another three kallikrein-like genes.

Figure 15 shows the genomic organization and partial genomic sequence of the KLK-L3 gene. Intronic sequences are not shown except for the splice junctions. Introns are shown with lower case letters and exons with capital letters. For the full sequence, see SEQ.ID. NO. 21. The start and stop codons are encircled and the exon –intron junctions are boxed. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are inside triangles. Putative polyadenylation signal is underlined.

Figure 16 is a plot of hydrophobicity and hydrophilicity, comparing the pattern of the KLK-L3 with that of the zyme gene. Note the hydrophobic region around the first twenty amino acids, likely representing the signal peptide.

Figure 17 is an alignment of the deduced amino acid sequence of KLK-L3 with members of the kallikrein multi-gene family. Genes are (from top to bottom and in brackets is the GenBank accession #): PSA (P07288), KLK2 (P20151), KLK1 (NP002248), trypsinogen (P07477), KLK-L3 (AF135026), trypsin-like serine protease (TLSP) (BAA33404), neuropsin (BAA28673), zyme (Q92876), human stratum corneum chymotryptic enzyme (HSCCE) (AAD49718), and/prostase/KLK-L1 (AAD21581). (See SEQ.ID. NOs. 78 to 84). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are bold and in italics, and the 29 invariant serine protease residues are denoted by (♦). Cysteine residues are marked by (•). Conserved areas around the catalytic triad are highlited in black. The arrow heads (▲) represent the potential cleavage sites. The dotted area represents the kallikrein loop sequence.

Figure 18 is a dendrogram of the predicted phylogenetic tree for some serine proteases and kallikrein genes. Neighbor-joining/UPGMA method was used to align KLK-L3 with other members of the kallikrein gene family. Gene names and accession numbers are listed in Figure 17. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L3 gene in one group with TLSP, neuropsin, and NES 1 genes. KLK-L4 (SEQ.ID.NO. 43) lies further telomeric to TLSP (21).

Figure 19 is a blot showing tissue expression of the KLK-L3 gene as determined by RT-PCR. Actin and PSA are control genes.

Figure 20 shows hormonal regulation of the KLK-L3 gene in the BT-474 breast carcinoma cell line. DHT = dihydrotestosterone. Steroids were at 10^{-8} M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins),

are control genes. KLK-L3 is upregulated by progestins, estrogens and androgens, in that order.

Figure 21 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2,neuropsin, NES1, and KLK-L3 genes. Exons are shown by black boxes and introns by the connecting lines. Arrowheads show the start codon, and arrows show the stop codon. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs.

Figure 22 shows a comparative genomic structure of the ESTs (Table 16), the clone from The German Genome Project, and the long form of KLK-L4. Exons are represented by solid bars and introns by the connecting lines. Exon numbers on top of solid bars refer to GenBank submission #AF135024. The EST IDs represent GenBank accession numbers. Asterisks represent the positions of stop codons. Horizontal arrows indicate the direction of the PCR primers (described in Table 15) and arrowheads their position along the exons. Vertical dotted lines show alignment of identical fragments.

Figure 23 shows tissue expression of the KLK-L4 gene as determined by RT-PCR. Actin and PSA are control genes. KLK-L4 is highly expressed in breast, prostate, salivary gland and testis.

Figure 24 in the Upper Panel is a Diagram showing the comparative genomic structure of the long KLK-L4 form and the short KLK-L4 variant. Exons are represented by boxes and introns by the connecting lines. Exon numbers refer to SEQ. ID. NO. 43 and GenBank Accession No. AF135024. The black region indicates the extra fragment (214 bp) that is found in the long, but not in the short form of the gene. The positions of the stop codons of the two forms are marked with asterisks. Frame shifting occurs as a result of utilization of an alternative splice site, and a stop codon is generated at the beginning of exon 4 in the short form. The Lower Panel shows PCR products of the amplification of the KLK-L4 gene using L4-R1 and L4-X1 primers (Figure 22 and Table 15). Note the predominant long form and a minor band representing the short form of KLK-L4 mRNA. (M); Markers with sizes in bp shown on the left. Tissues used: (1), salivary gland; (2), mammary gland; (3), prostate; (4), testis; (5), uterus; (6), breast cancer tissue; (7), negative control.

Figure 25 shows the genomic organization and partial genomic sequence of the KLK-L4 gene. Intronic sequences are not shown except for the splice junction areas. Introns are shown with lower case letters and exons with capital letters. For full sequence, see SEQ. ID. NO.43 or GenBank Accession #AF135024. The start and stop codons are encircled and the exon –intron junctions are underlined. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are boxed. The putative polyadenylation signal is underlined.

Figure 26 is a plot of hydrophobicity and hydrophilicity of the KLK-L4 protein, as compared with the glandular kallikrein gene 2 (KLK2). Note the hydrophobic region at the amino terminus, suggesting presence of a signal peptide.

Figure 27 shows an alignment of the deduced amino acid sequence of KLK-L4 with members of the kallikrein multi-gene family. Genes are (from top to bottom, and in brackets are the GenBank accession

#): KLK-L1/prostase (AAD21581), enamel matrix serine proteinase 1 (EMSP) (NP_004908), KLK-L2 (AF135028), PSA (P07288), KLK2 (P20151), KLK1 (NP_002248), trypsinogen (P07477), zyme (Q92876), KLK-L4 (AF135024), trypsin-like serine protease (TLSP) (BAA33404), KLK-L3 (AF135026),neuropsin (BAA28673), and the normal epithelial cell-specific 1 gene (NES1) (O43240). (See SEQ.ID. NOs. 78-88). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are typed in bold, and conserved motifs around them are highlighted in grey.The 29 invariant serine protease residues are denoted by (•), and the cysteine residues by (♦). The predicted cleavage sites are indicated by (♠). The dotted area represents the kallikrein loop sequence. The trypsin-like cleavage pattern of KLK-L4 with the D residue, is indicated by (✪).

Figure 28 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4.Genes are represented by horizontal arrows denoting the direction of the coding sequence. Their lengths are shown on top of each arrow. Distances between genes are mentioned in base pairs below the arrows. The distance between KLK1 and PSA is not accurately known. For gene names, see under abbreviations.

Figure 29 shows is a dendrogram of the predicted phylogenetic tree for some kallikrein and serine protease genes. The neighbor-joining/UPGMA method was used to align KLK-L4 with other serine proteases and members of the kallikrein gene family. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L4 gene in one group with zyme, NES1, neuropsin, KLK-L3, and TLSP. Other serine proteases were aligned in different groups, as shown.

Figure 30 is a blot showing the hormonal regulation of the KLK-L4 gene in the BT-474 breast carcinoma cell line. DHT = dihydrotestosterone. Steroids were added at 10^{-8} M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins) are control genes. KLK-L4 is up-regulated by androgens and progestins and to a lesser extent by estrogens. H_2O was used to check for PCR specificity in all PCR reactions. For more details, see text.

Figure 31 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, and KLK-L4 genes. Exons are shown by black boxes and introns by the connecting lines. The arrowhead shows the start codons and the arrow the stop codons. Letters above boxes indicate the relative positions of the amino acids of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I, the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs. The question mark indicates the possibility of more untranslated bases.

Figure 32 is a diagram showing the comparative genomic structure of the three splice forms of KLK-L5; the classical kallikrein form, related protein-1, and related protein-2. Exons are represented by solid bars and introns by the connecting lines. Exon numbers refer to SEQ.ID. NO.56 and GenBank Accession #AF135025. Start codons are represented by the inverted arrowhead (▼) and stop codons are represented by asterisks (✱). Primer locations are represented by vertical arrowheads (▲) and their

directions by horizontal arrows. For primer sequences and codes see Table 17 and SEQ.ID. NOs. 61-64, and 9-12.

Figure 33 shows the genomic organization and partial genomic sequence of the KLK-L5 gene. Intronic sequences are not shown except for short sequences around the splice junctions. Introns are shown with lower case letters and exons with capital letters. For full sequence, see SEQ.ID.NO. 56. The start and stop codons are encircled and the exon –intron junctions are underlined. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are boxed. Putative polyadenylation signal is underlined. The extra intron of the related protein-1 form is represented by non-bold capital letters between brackets. When this intron is spliced, the frame continues with codon AAC (asparagine, N, instead of lysine, K) until it encounters the stop codon TAA (encircled).

Figure 34 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, KLK-L4 and KLK-L5 genes. Exons are shown by solid bars and introns by the connecting lines. Arrowhead marks the site of the start codon, and the arrow represents the stop codon. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs. Question marks indicate that exact length is not accurately known. .

Figure 35 is a plot of hydrophobicity and hydrophilicity of KLK-L5 protein compared to prostate specific antigen (PSA). The hydrophobic N-terminus may harbor a signal and activation peptide.

Figure 36 shows an alignment of the deduced amino acid sequence of KLK-L5 with members of the kallikrein multigene family. (See SEQ.ID. NOs. 78-81, 83, 84). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are represented by bold letters, and the 29 invariant serine protease residues are marked with (•). The cysteine residues are marked by (♦). Conserved areas are highlighted in grey. The predicted cleavage sites in signal peptide are indicated by (♠). The dotted area represents the kallikrein loop sequence. A vertical arrow marks the trypsin like cleavage site.

Figure 37 is a dendrogram of the predicted phylogenetic tree for some serine proteases and other kallikrein proteins. Neighbor-joining/UPGMA method was used to align KLK-L5 with other serine proteases and members of the kallikrein gene family. The tree grouped the classical kallikreins (hK1, hK2, and PSA) together and aligned the KLK-L5 protein in one group with NES1 and neuropsin. Other serine proteases were aligned in different groups.

Figure 38 shows tissue expression of the KLK-L5 gene as determined by RT-PCR. The upper band (905 base pairs, bp) is the classical form (see Figure 32, the middle (776 bp) the related protein-1, and the lower band (644 bp) the related protein-2. For splice variant discussion see text. The primers used were L5-F2 and L5-R2, as shown in Table 17.

Figure 39 shows hormonal regulation of the KLK-L5 gene in the LnCaP prostatic carcinoma cell line, BT-474 and T-47D breast carcinoma cell lines. Steroids were at 10^{-8} M final concentration. Actin (not

regulated by steroid hormones) was used as a control gene. Note detection of three isoforms only in LNCaP.

Figure 40 shows the expression of the KLK-L5 gene in breast cancer (1-17) and normal (18) tissues. Note complete absence of expression in 12 cancer tissues. For isoforms see also Figure 38.

Figure 41 shows the full structure of a KLK-L6 nucleic acid molecule;

Figure 42 is a plot of hydrophobicity and hydrophilicity of KLK-L6 protein compared to prostate specific antigen (PSA).

Figure 43 shows an alignment of the deduced amino acid sequence of KLK-L6 with members of the kallikrein multigene family. (See SEQ.ID. NOs. 78-81, 83, 84). Dashes represent gaps to bring the sequences to better alignment.

Figure 44 is a dendrogram of the predicted phylogenetic tree for some serine proteases and other kallikrein proteins. Neighbor-joining/UPGMA method was used to align KLK-L6 with other serine proteases and members of the kallikrein gene family.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See for example, Sambrook, Fritsch, & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M..J. Gait ed. 1984); Nucleic Acid Hybridization B.D. Hames & S.J. Higgins eds. (1985); Transcription and Translation B.D. Hames & S.J. Higgins eds (1984); Animal Cell Culture R.I. Freshney, ed. (1986); Immobilized Cells and enzymes IRL Press, (1986); and B. Perbal, A Practical Guide to Molecular Cloning (1984).

1. <u>Nucleic Acid Molecules of the Invention</u>

As hereinbefore mentioned, the invention provides an isolated nucleic acid molecule having a sequence encoding a KLK-L Protein. The term "isolated" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical reactants, or other chemicals when chemically synthesized. An "isolated" nucleic acid may also be free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid molecule) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded. In an embodiment, a nucleic acid molecule encodes a KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, preferably a nucleic acid molecule comprising a nucleic acid sequence as shown in SEQ.ID.NO. 1, 13, 21, 43, 56, or 65.

The invention includes nucleic acid sequences complementary to a nucleic acid encoding a KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, preferably the nucleic acid sequences complementary to a full nucleic acid sequence shown in SEQ.ID.NO. 1, 13, 21, 43, 56, or 65.

The invention includes nucleic acid molecules having substantial sequence identity or homology to nucleic acid sequences of the invention or encoding proteins having substantial identity or similarity to the amino acid sequence shown in in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67. Preferably, the nucleic acids have substantial sequence identity for example at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% nucleic acid identity; more preferably 90% nucleic acid identity; and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity. "Identity" as known in the art and used herein, is a relationship between two or more amino acid sequences or two or more nucleic acid sequences, as determined by comparing the sequences. It also refers to the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. Identity and similarity are well known terms to skilled artisans and they can be calculated by conventional methods (for example see Computational Molecular Biology, Lesk, A.M. ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W. ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M. and Griffin, H.G. eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G. Acadmeic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J. eds. M. Stockton Press, New York, 1991, Carillo, H. and Lipman, D., SIAM J. Applied Math. 48:1073, 1988). Methods which are designed to give the largest match between the sequences are generally preferred. Methods to determine identity and similarity are codified in publicly available computer programs including the GCG program package (Devereux J. et al., Nucleic Acids Research 12(1): 387, 1984); BLASTP, BLASTN, and FASTA (Atschul, S.F. et al. J. Molec. Biol. 215: 403-410, 1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al. NCBI NLM NIH Bethesda, Md. 20894; Altschul, S. et al. J. Mol. Biol. 215: 403-410, 1990).

Isolated nucleic acid molecules encoding a KLK-L Protein, and having a sequence which differs from a nucleic acid sequence of the invention due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent proteins (e.g., a KLK-L Protein) but differ in sequence from the sequence of a KLK-L Protein due to degeneracy in the genetic code. As one example, DNA sequence polymorphisms within the nucleotide sequence of a KLK-L Protein may result in silent mutations which do not affect the amino acid sequence. Variations in one or more nucleotides may exist among individuals within a population due to natural allelic variation. Any and all such nucleic acid variations are within the scope of the invention. DNA sequence polymorphisms may also occur which lead to changes in the amino acid sequence of a KLK-L Protein. These amino acid polymorphisms are also within the scope of the present invention.

Another aspect of the invention provides a nucleic acid molecule which hybridizes under stringent conditions, preferably high stringency conditions to a nucleic acid molecule which comprises a sequence which encodes a KLK-L Protein having an amino acid sequence shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C,

followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

It will be appreciated that the invention includes nucleic acid molecules encoding a KLK-L Related Protein including truncations of a KLK-L Protein, and analogs of a KLK-L Protein as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention. (See for example, splice variants of KLK-L5, SEQ.ID.NO. 58, 59, and 60.)

An isolated nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of a nucleic acid sequence of the invention. The labeled nucleic acid probe is used to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). For example, a cDNA library can be used to isolate a cDNA encoding a KLK-L Related Protein by screening the library with the labeled probe using standard techniques. Alternatively, a genomic DNA library can be similarly screened to isolate a genomic clone encompassing a gene encoding a KLK-L Related Protein. Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding a KLK-L Related Protein using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence of the invention for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a KLK-L Related Protein into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a KLK-L Related Protein. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by conventional techniques.

Nucleic acid molecules of the invention may be chemically synthesized using standard techniques. Methods of chemically synthesizing polydeoxynucleotides are known, including but not limited to solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a KLK-L Related Protein can be accomplished by expressing the cDNA in an appropriate host cell by standard techniques, and testing the expressed protein in the methods described herein. A cDNA encoding a KLK-L Related Protein can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

The initiation codon and untranslated sequences of a KLK-L Related Protein may be determined using computer software designed for the purpose, such as PC/Gene (IntelliGenetics Inc., Calif.). The intron-exon structure and the transcription regulatory sequences of a gene encoding a KLK-L Related Protein may be confirmed by using a nucleic acid molecule of the invention encoding a KLK-L Related Protein to probe a genomic DNA clone library. Regulatory elements can be identified using standard techniques. The function of the elements can be confirmed by using these elements to express a reporter gene such as the lacZ gene which is operatively linked to the elements. These constructs may be introduced into cultured cells using conventional procedures or into non-human transgenic animal models. In addition to identifying regulatory elements in DNA, such constructs may also be used to identify nuclear proteins interacting with the elements, using techniques known in the art.

In a particular embodiment of the invention, the nucleic acid molecules isolated using the methods described herein are mutant *klk-l* gene alleles. The mutant alleles may be isolated from individuals either known or proposed to have a genotype which contributes to the symptoms of for example, cancer (e.g.. breast, testicular, brain, colon, and prostate cancer). Mutant alleles and mutant allele products may be used in therapeutic and diagnostic methods described herein. For example, a cDNA of a mutant *klk-l* gene may be isolated using PCR as described herein, and the DNA sequence of the mutant allele may be compared to the normal allele to ascertain the mutation(s) responsible for the loss or alteration of function of the mutant gene product. A genomic library can also be constructed using DNA from an individual suspected of or known to carry a mutant allele, or a cDNA library can be constructed using RNA from tissue known, or suspected to express the mutant allele. A nucleic acid encoding a normal *klk-l* gene or any suitable fragment thereof, may then be labeled and used as a probe to identify the corresponding mutant allele in such libraries. Clones containing mutant sequences can be purified and subjected to sequence analysis. In addition, an expression library can be constructed using cDNA from RNA isolated from a tissue of an individual known or suspected to express a mutant *klk-l* allele. Gene products made by the putatively mutant tissue may be expressed and screened, for example using antibodies specific for a KLK-L Related Protein as described herein. Library clones identified using the antibodies can be purified and subjected to sequence analysis.

The sequence of a nucleic acid molecule of the invention, or a fragment of the molecule, may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

2. Proteins of the Invention

An amino acid sequence of a KLK-L Protein comprises a sequence as shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.

In addition to proteins comprising an amino acid sequence as shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, the proteins of the present invention include truncations of a KLK-L Protein, analogs of a KLK-L Protein, and proteins having sequence identity or similarity to a KLK-L Protein, and truncations thereof as described herein (i.e.included in KLK-L Related Proteins). Truncated proteins may comprise peptides of between 3 and 70 amino acid residues, ranging in size from a tripeptide to a 70 mer polypeptide.

The truncated proteins may have an amino group (-NH2), a hydrophobic group (for example, carbobenzoxyl, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl (PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated proteins may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of a KLK-L Protein, and/or truncations thereof as described herein, which may include, but are not limited to a KLK-L Protein, containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of a KLK-L Protein amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog is preferably functionally equivalent to a KLK-L Protein. Non-conserved substitutions involve replacing one or more amino acids of the KLK-L Protein amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into a KLK-L Protein. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length.

Deletions may consist of the removal of one or more amino acids, or discrete portions from a KLK-L Protein sequence. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 20 to 40 amino acids.

The proteins of the invention include proteins with sequence identity or similarity to a KLK-L Protein and/or truncations thereof as described herein. Such KLK-L Proteins include proteins whose amino acid sequences are comprised of the amino acid sequences of KLK-L Protein regions from other species that hybridize under selected hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain a KLK-L Protein. These proteins will generally have the same regions which are characteristic of a KLK-L Protein. Preferably a protein will have substantial sequence identity for example, about 30%, 35%, 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, or 85% identity, preferably 90% identity, more preferably at least 95%, 96%, 97%, 98%, or 99% identity, and most preferably 98% identity with an amino acid sequence shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57,

58, 59, 60, 66, or 67.

A percent amino acid sequence homology, similarity or identity is calculated as the percentage of aligned amino acids that match the reference sequence using known methods as described herein.

The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as a protein of the invention, but the isoform has a different molecular structure. Isoforms contemplated by the present invention preferably have the same properties as a protein of the invention as described herein.

The present invention also includes KLK-L Related Proteins conjugated with a selected protein, or a marker protein (see below) to produce fusion proteins. Additionally, immunogenic portions of a KLK-L Protein and a KLK-L Protein Related Protein are within the scope of the invention.

A KLK-L Related Protein of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which encodes a KLK-L Related Protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. The necessary regulatory sequences may be supplied by the native KLK-L Protein and/or its flanking regions.

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is linked to a regulatory sequence in a manner which allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to the nucleic acid sequence of a protein of the invention or a fragment thereof. Regulatory sequences linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance a viral promoter and/or enhancer, or regulatory sequences can be chosen which direct tissue or cell type specific expression of antisense RNA.

The recombinant expression vectors of the invention may also contain a marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. The markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

The recombinant expression vectors may be introduced into host cells to produce a transformant host cell. "Transformant host cells" include host cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" encompass the introduction of a nucleic acid (e.g. a vector) into a cell by one of many standard techniques. Prokaryotic cells can be transformed with a nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. A nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells, or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

A host cell may also be chosen which modulates the expression of an inserted nucleic acid sequence, or modifies (e.g. glycosylation or phosphorylation) and processes (e.g. cleaves) the protein in a desired fashion. Host systems or cell lines may be selected which have specific and characteristic mechanisms for post-translational processing and modification of proteins. For example, eukaryotic host cells including CHO, VERO, BHK, HeLA, COS, MDCK, 293, 3T3, and WI38 may be used. For long-term high-yield stable expression of the protein, cell lines and host systems which stably express the gene product may be engineered.

Host cells and in particular cell lines produced using the methods described herein may be particularly useful in screening and evaluating compounds that modulate the activity of a KLK-L Related Protein.

The proteins of the invention may also be expressed in non-human transgenic animals including but not limited to mice, rats, rabbits, guinea pigs, micro-pigs, goats, sheep, pigs, non-human primates (e.g. baboons, monkeys, and chimpanzees) [see Hammer et al. (Nature 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866)]. Procedures known in the art may be

used to introduce a nucleic acid molecule of the invention encoding a KLK-L Related Protein into animals to produce the founder lines of transgenic animals. Such procedures include pronuclear microinjection, retrovirus mediated gene transfer into germ lines, gene targeting in embryonic stem cells, electroporation of embryos, and sperm-mediated gene transfer.

The present invention contemplates a transgenic animal that carries the *KLK-L* gene in all their cells, and animals which carry the transgene in some but not all their cells. The transgene may be integrated as a single transgene or in concatamers. The transgene may be selectively introduced into and activated in specific cell types (See for example, Lasko et al, 1992 Proc. Natl. Acad. Sci. USA 89: 6236). The transgene may be integrated into the chromosomal site of the endogenous gene by gene targeting. The transgene may be selectively introduced into a particular cell type inactivating the endogenous gene in that cell type (See Gu et al Science 265: 103-106).

The expression of a recombinant KLK-L Related Protein in a transgenic animal may be assayed using standard techniques. Initial screening may be conducted by Southern Blot analysis, or PCR methods to analyze whether the transgene has been integrated. The level of mRNA expression in the tissues of transgenic animals may also be assessed using techniques including Northern blot analysis of tissue samples, *in situ* hybridization, and RT-PCR. Tissue may also be evaluated immunocytochemically using antibodies against KLK-L Protein.

Proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

N-terminal or C-terminal fusion proteins comprising a KLK-L Related Protein of the invention conjugated with other molecules, such as proteins, may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of a KLK-L Related Protein, and the sequence of a selected protein or marker protein with a desired biological function. The resultant fusion proteins contain KLK-L Protein fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

3. __Antibodies__

KLK-L Related Proteins of the invention can be used to prepare antibodies specific for the proteins. Antibodies can be prepared which bind a distinct epitope in an unconserved region of the protein. An unconserved region of the protein is one that does not have substantial sequence homology to other proteins. A region from a conserved region such as a well-characterized domain can also be used to prepare an antibody to a conserved region of a KLK-L Related Protein. Antibodies having specificity for a KLK-L Related Protein may also be raised from fusion proteins created by expressing fusion proteins in bacteria as described herein.

The invention can employ intact monoclonal or polyclonal antibodies, and immunologically active fragments (e.g. a Fab, $(Fab)_2$ fragment, or Fab expression library fragments and epitope-binding fragments

thereof), an antibody heavy chain, and antibody light chain, a genetically engineered single chain Fv molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

4. <u>Applications of the Nucleic Acid Molecules, KLK-L Related Proteins, and Antibodies of the Invention</u>

The nucleic acid molecules, KLK-L Related Proteins, and antibodies of the invention may be used in the prognostic and diagnostic evaluation of cancer (e.g. breast, testicular, and prostate cancer) or other conditions, and the identification of subjects with a predisposition to cancer (Section 4.1.1 and 4.1.2). Methods for detecting nucleic acid molecules and KLK-L Related Proteins of the invention, can be used to monitor conditions including cancer, by detecting KLK-L Related Proteins and nucleic acid molecules encoding KLK-L Related Proteins. It would also be apparent to one skilled in the art that the methods described herein may be used to study the developmental expression of KLK-L Related Proteins and, accordingly, will provide further insight into the role of KLK-L Related Proteins. The applications of the present invention also include methods for the identification of compounds that modulate the biological activity of *KLK-L* or KLK-L Related Proteins (Section 4.2). The compounds, antibodies etc. may be used for the treatment of cancer (Section 4.3).

4.1 <u>Diagnostic Methods</u>

A variety of methods can be employed for the diagnostic and prognostic evaluation of conditions including cancer (e.g. breast, testicular, and prostate cancer), and the identification of subjects with a predisposition to such conditions. Such methods may, for example, utilize nucleic acid molecules of the invention, and fragments thereof, and antibodies directed against KLK-L Related Proteins, including peptide fragments. In particular, the nucleic acids and antibodies may be used, for example, for: (1) the detection of the presence of *KLK-L* mutations, or the detection of either over- or under-expression of *KLK-L* mRNA relative to a non-disorder state or the qualitative or quantitative detection of alternatively spliced forms of *KLK-L* transcripts which may correlate with certain conditions or susceptibility toward such conditions; and (2) the detection of either an over- or an under-abundance of KLK-L Related Proteins relative to a non- disorder state or the presence of a modified (e.g., less than full length) KLK-L Protein which correlates with a disorder state, or a progression toward a disorder state.

The methods described herein may be performed by utilizing pre-packaged diagnostic kits comprising at least one specific *KLK-L* nucleic acid or antibody described herein, which may be conveniently used, e.g., in clinical settings, to screen and diagnose patients and to screen and identify those individuals exhibiting a predisposition to developing a disorder.

Nucleic acid-based detection techniques are described, below, in Section 4.1.1. Peptide detection techniques are described, below, in Section 4.1.2. The samples that may be analyzed using the methods of the invention include those which are known or suspected to express *KLK-L* or contain KLK-L Related Proteins. The samples may be derived from a patient or a cell culture, and include but are not limited to

biological fluids, tissue extracts, freshly harvested cells, and lysates of cells which have been incubated in cell cultures.

Oligonucleotides or longer fragments derived from any of the nucleic acid molecules of the invention may be used as targets in a microarray. The microarray can be used to simultaneously monitor the expression levels of large numbers of genes and to identify genetic variants, mutations, and polymorphisms. The information from the microarray may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose a disorder, and to develop and monitor the activities of therapeutic agents.

The preparation, use, and analysis of microarrays are well known to a person skilled in the art. (See, for example, Brennan, T. M. et al. (1995) U.S. Pat. No. 5,474,796; Schena, et al. (1996) Proc. Natl. Acad. Sci. 93:10614-10619; Baldeschweiler et al. (1995), PCT Application WO95/251116; Shalon, D. et al. (I 995) PCT application WO95/35505; Heller, R. A. et al. (1997) Proc. Natl. Acad. Sci. 94:2150-2155; and Heller, M. J. et al. (1997) U.S. Pat. No. 5,605,662.)

4.1.1 Methods for Detecting Nucleic Acid Molecules of the Invention

The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences of the invention in samples. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding at least 5 sequential amino acids from regions of a KLK-L Protein, preferably they comprise 15 to 30 nucleotides. A nucleotide probe may be labeled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labeled antibody, fluorescent compounds, enzymes, antibodies specific for a labeled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labeled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic acid probes may be used to detect genes, preferably in human cells, that encode KLK-L Related Proteins. The nucleotide probes may also be useful in the diagnosis of cancer; in monitoring the progression of cancer; or monitoring a therapeutic treatment.

The probe may be used in hybridization techniques to detect genes that encode KLK-L Related Proteins. The technique generally involves contacting and incubating nucleic acids (e.g. recombinant DNA molecules, cloned genes) obtained from a sample from a patient or other cellular source with a probe of the present invention under conditions favorable for the specific annealing of the probes to complementary sequences in the nucleic acids. After incubation, the non-annealed nucleic acids are removed, and the presence of nucleic acids that have hybridized to the probe if any are detected.

The detection of nucleic acid molecules of the invention may involve the amplification of specific gene sequences using an amplification method such as PCR, followed by the analysis of the amplified molecules using techniques known to those skilled in the art. Suitable primers can be routinely designed

by one of skill in the art.

Genomic DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving *klk- l* structure, including point mutations, insertions, deletions, and chromosomal rearrangements. For example, direct sequencing, single stranded conformational polymorphism analyses, heteroduplex analysis, denaturing gradient gel electrophoresis, chemical mismatch cleavage, and oligonucleotide hybridization may be utilized.

Genotyping techniques known to one skilled in the art can be used to type polymorphisms that are in close proximity to the mutations in a *klk-l* gene. The polymorphisms may be used to identify individuals in families that are likely to carry mutations. If a polymorphism exhibits linkage disequalibrium with mutations in a *klk-l* gene, it can also be used to screen for individuals in the general population likely to carry mutations. Polymorphisms which may be used include restriction fragment length polymorphisms (RFLPs), single-base polymorphisms, and simple sequence repeat polymorphisms (SSLPs).

A probe of the invention may be used to directly identify RFLPs. A probe or primer of the invention can additionally be used to isolate genomic clones such as YACs, BACs, PACs, cosmids, phage or plasmids. The DNA in the clones can be screened for SSLPs using hybridization or sequencing procedures.

Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of *klk-l* expression. For example, RNA may be isolated from a cell type or tissue known to express *klk-l* and tested utilizing the hybridization (e.g. standard Northern analyses) or PCR techniques referred to herein. The techniques may be used to detect differences in transcript size which may be due to normal or abnormal alternative splicing. The techniques may be used to detect quantitative differences between levels of full length and/or alternatively splice transcripts detected in normal individuals relative to those individuals exhibiting cancer symptoms or other disease conditions.

The primers and probes may be used in the above described methods *in situ* i.e directly on tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections.

4.1.2 <u>Methods for Detecting KLK-L Related Proteins</u>

Antibodies specifically reactive with a KLK-L Related Protein, or derivatives, such as enzyme conjugates or labeled derivatives, may be used to detect KLK-L Related Proteins in various samples (e.g. biological materials). They may be used as diagnostic or prognostic reagents and they may be used to detect abnormalities in the level of KLK-L Related Proteins expression, or abnormalities in the structure, and/or temporal, tissue, cellular, or subcellular location of a KLK-L Related Protein. Antibodies may also be used to screen potentially therapeutic compounds *in vitro* to determine their effects on cancer, and other conditions. *In vitro* immunoassays may also be used to assess or monitor the efficacy of particular therapies. The antibodies of the invention may also be used *in vitro* to determine the level of *KLK-L* expression in cells genetically engineered to produce a KLK-L Related Protein.

The antibodies may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of a KLK-L Related Protein and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation,

latex agglutination, hemagglutination, and histochemical tests. The antibodies may be used to detect and quantify KLK-L Related Proteins in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect a KLK-L Related Protein, to localize it to particular cells and tissues, and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect a KLK-L Related Protein. Generally, an antibody of the invention may be labeled with a detectable substance and a KLK-L Related Protein may be localised in tissues and cells based upon the presence of the detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol; enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached via spacer arms of various lengths to reduce potential steric hindrance. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

The antibody or sample may be immobilized on a carrier or solid support which is capable of immobilizing cells, antibodies etc. For example, the carrier or support may be nitrocellulose, or glass, polyacrylamides, gabbros, and magnetite. The support material may have any possible configuration including spherical (e.g. bead), cylindrical (e.g. inside surface of a test tube or well, or the external surface of a rod), or flat (e.g. sheet, test strip). Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against KLK-L Related Protein. By way of example, if the antibody having specificity against a KLK-L Related Protein is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labeled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, a KLK-L Related Protein may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

4.2 <u>Methods for Identifying or Evaluating Substances/Compounds</u>

The methods described herein are designed to identify substances that modulate the biological activity of a KLK-L Related Protein including substances that bind to KLK-L Related Proteins, or bind to other proteins that interact with a KLK-L Related Protein, to compounds that interfere with, or enhance the interaction of a KLK-L Related Protein and substances that bind to the KLK-L Related Protein or other proteins that interact with a KLK-L Related Protein. Methods are also utilized that identify compounds that bind to *KLK-L* regulatory sequences.

The substances and compounds identified using the methods of the invention include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, $F(ab)_2$, and Fab expression library fragments, and epitope-binding fragments thereof)], and small organic or inorganic molecules. The substance or compound may be an endogenous physiological compound or it may be a natural or synthetic compound.

Substances which modulate a KLK-L Related Protein can be identified based on their ability to bind to a KLK-L Related Protein. Therefore, the invention also provides methods for identifying substances which bind to a KLK-L Related Protein. Substances identified using the methods of the invention may be isolated, cloned and sequenced using conventional techniques. A substance that associates with a polypeptide of the invention may be an agonist or antagonist of the biological or immunological activity of a polypeptide of the invention.

The term "agonist", refers to a molecule that increases the amount of, or prolongs the duration of, the activity of the polypeptide. The term "antagonist" refers to a molecule which decreases the biological or immunological activity of the polypeptide. Agonists and antagonists may include proteins, nucleic acids, carbohydrates, or any other molecules that associate with a polypeptide of the invention.

Substances which can bind with a KLK-L Related Protein may be identified by reacting a KLK-L Related Protein with a test substance which potentially binds to a KLK-L Related Protein, under conditions which permit the formation of substance-KLK-L Related Protein complexes and removing and/or detecting the complexes. The complexes can be detected by assaying for substance-KLK-L Related Protein complexes, for free substance, or for non-complexed KLK-L Related Protein. Conditions which permit the formation of substance-KLK-L Related Protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-protein complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against KLK-L Related Protein or the substance, or labeled KLK-L Related Protein, or a labeled substance may be utilized. The antibodies, proteins, or substances may be labeled with a detectable substance as described above.

A KLK-L Related Protein, or the substance used in the method of the invention may be insolubilized. For example, a KLK-L Related Protein, or substance may be bound to a suitable carrier such as agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical

methods, for example, cyanogen bromide coupling.

The invention also contemplates a method for evaluating a compound for its ability to modulate the biological activity of a KLK-L Related Protein of the invention, by assaying for an agonist or antagonist (i.e. enhancer or inhibitor) of the binding of a KLK-L Related Protein with a substance which binds with a KLK-L Related Protein. The basic method for evaluating if a compound is an agonist or antagonist of the binding of a KLK-L Related Protein and a substance that binds to the protein, is to prepare a reaction mixture containing the KLK-L Related Protein and the substance under conditions which permit the formation of substance-KLK-L Related Protein complexes, in the presence of a test compound. The test compound may be initially added to the mixture, or may be added subsequent to the addition of the KLK-L Related Protein and substance. Control reaction mixtures without the test compound or with a placebo are also prepared. The formation of complexes is detected and the formation of complexes in the control reaction but not in the reaction mixture indicates that the test compound interferes with the interaction of the KLK-L Related Protein and substance. The reactions may be carried out in the liquid phase or the KLK-L Related Protein, substance, or test compound may be immobilized as described herein. The ability of a compound to modulate the biological activity of a KLK-L Related Protein of the invention may be tested by determining the biological effects on cells.

It will be understood that the agonists and antagonists i.e. inhibitors and enhancers that can be assayed using the methods of the invention may act on one or more of the binding sites on the protein or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of KLK-L Related Protein with a substance which is capable of binding to the KLK-L Related Protein. Thus, the invention may be used to assay for a compound that competes for the same binding site of a KLK-L Related Protein.

The invention also contemplates methods for identifying compounds that bind to proteins that interact with a KLK-L Related Protein. Protein-protein interactions may be identified using conventional methods such as co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Methods may also be employed that result in the simultaneous identification of genes which encode proteins interacting with a KLK-L Related Protein. These methods include probing expression libraries with labeled KLK-L Related Protein.

Two-hybrid systems may also be used to detect protein interactions *in vivo*. Generally, plasmids are constructed that encode two hybrid proteins. A first hybrid protein consists of the DNA-binding domain of a transcription activator protein fused to a KLK-L Related Protein, and the second hybrid protein consists of the transcription activator protein's activator domain fused to an unknown protein encoded by a cDNA which has been recombined into the plasmid as part of a cDNA library. The plasmids are transformed into a strain of yeast (e.g. *S. cerevisiae*) that contains a reporter gene (e.g. lacZ, luciferase, alkaline phosphatase, horseradish peroxidase) whose regulatory region contains the transcription activator's binding site. The hybrid proteins alone cannot activate the transcription of the reporter gene. However,

interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

It will be appreciated that fusion proteins may be used in the above-described methods. In particular, KLK-L Related Proteins fused to a glutathione-S-transferase may be used in the methods.

The reagents suitable for applying the methods of the invention to evaluate compounds that modulate a KLK-L Related Protein may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

4.3 Compositions and Treatments

The proteins of the invention, substances or compounds identified by the methods described herein, antibodies, and antisense nucleic acid molecules of the invention may be used for modulating the biological activity of a KLK-L Related Protein, and they may be used in the treatment of conditions such as cancer (e.g. prostate, testicular, brain, uterine, thymus, ovarian, colon, ovarian, or breast cancer). Accordingly, the substances, antibodies, peptides, and compounds may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the active substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The active substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of a pharmaceutical composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the substance from the action of enzymes, acids and other natural conditions that may inactivate the substance.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the active substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

Based upon their homology to genes encoding kallikrein, nucleic acid molecules of the invention may be also useful in the treatment of conditions such as hypertension, cardiac hypertrophy, arthritis, inflammatory disorders, neurological disorders, and blood clotting disorders.

Vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids, may be used to deliver nucleic acid molecules to a targeted organ, tissue, or cell population. Methods well known to those skilled in the art may be used to construct recombinant vectors which will express antisense nucleic acid molecules of the invention. (See, for example, the techniques described in Sambrook et al (supra) and Ausubel et al (supra)).

The nucleic acid molecules comprising full length cDNA sequences and/or their regulatory elements enable a skilled artisan to use sequences encoding a protein of the invention as an investigative tool in sense (Youssoufian H and H F Lodish 1993 Mol Cell Biol 13:98-104) or antisense (Eguchi et al (1991) Annu Rev Biochem 60:631-652) regulation of gene function. Such technology is well known in the art, and sense or antisense oligomers, or larger fragments, can be designed from various locations along the coding or control regions.

Genes encoding a protein of the invention can be turned off by transfecting a cell or tissue with vectors which express high levels of a desired KLK-L-encoding fragment. Such constructs can inundate cells with untranslatable sense or antisense sequences. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until all copies are disabled by endogenous nucleases.

Modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA or PNA, to the regulatory regions of a gene encoding a protein of the invention, ie, the promoters, enhancers, and introns. Preferably, oligonucleotides are derived from the transcription initiation site, eg, between -10 and +10 regions of the leader sequence. The antisense molecules may also be designed so that they block translation of mRNA by preventing the transcript from binding to ribosomes. Inhibition may also be achieved using "triple helix" base-pairing methodology. Triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Therapeutic advances using triplex DNA were reviewed by Gee J E et al (In: Huber B E and B I Carr (1994) Molecular and Immunologic Approaches, Futura Publishing Co, Mt Kisco N.Y.).

Ribozymes are enzymatic RNA molecules that catalyze the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. The invention therefore contemplates engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding a protein of the invention.

Specific ribozyme cleavage sites within any potential RNA target may initially be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once the sites are identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets

may also be determined by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Methods for introducing vectors into cells or tissues include those methods discussed herein and which are suitable for *in vivo*, *in vitro* and *ex vivo* therapy. For *ex vivo* therapy, vectors may be introduced into stem cells obtained from a patient and clonally propagated for autologous transplant into the same patient (See U.S. Pat. Nos. 5,399,493 and 5,437,994). Delivery by transfection and by liposome are well known in the art.

The nucleic acid molecules disclosed herein may also be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including but not limited to such properties as the triplet genetic code and specific base pair interactions.

The invention also provides methods for studying the function of a polypeptide of the invention. Cells, tissues, and non-human animals lacking in expression or partially lacking in expression of a nucleic acid molecule or gene of the invention may be developed using recombinant expression vectors of the invention having specific deletion or insertion mutations in the gene. A recombinant expression vector may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a deficient cell, tissue, or animal.

Null alleles may be generated in cells, such as embryonic stem cells by deletion mutation. A recombinant gene may also be engineered to contain an insertion mutation that inactivates the gene. Such a construct may then be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, electroporation, injection etc. Cells lacking an intact gene may then be identified, for example by Southern blotting, Northern Blotting, or by assaying for expression of the encoded polypeptide using the methods described herein. Such cells may then be fused to embryonic stem cells to generate transgenic non-human animals deficient in a polypeptide of the invention. Germline transmission of the mutation may be achieved, for example, by aggregating the embryonic stem cells with early stage embryos, such as 8 cell embryos, *in vitro*; transferring the resulting blastocysts into recipient females and; generating germline transmission of the resulting aggregation chimeras. Such a mutant animal may be used to define specific cell populations, developmental patterns and *in vivo* processes, normally dependent on gene expression.

The invention thus provides a transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant expression vector that inactivates or alters a gene encoding a KLK-L Related Protein. In an embodiment the invention provides a transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant expression vector that inactivates or alters a gene encoding a KLK-L Related Protein resulting in a KLK-L Related Protein associated pathology. Further the invention provides a transgenic non-human mammal which doe not express a KLK-L Related Protein of the invention. In an embodiment, the invention provides a transgenic non-human mammal which doe not express a KLK-L Related Protein of the invention resulting in a KLK-L Related Protein associated pathology. A KLK-L Related Protein pathology refers to a phenotype observed for a KLK-L Related Protein homozygous mutant.

A transgenic non-human animal includes but is not limited to mouse, rat, rabbit, sheep, hamster, dog, guinea pig, micro-pig, pig, cat, goat, and non-human primates, preferably mouse.

The invention also provides a transgenic non-human animal assay system which provides a model system for testing for an agent that reduces or inhibits a pathology associated with an KLK-L Related Protein, preferably a KLK-L Related Protein associated pathology, comprising:

(a) administering the agent to a transgenic non-human animal of the invention; and

(b) determining whether said agent reduces or inhibits the pathology (e.g. KLK-L Related Protein associated pathology) in the transgenic non-human animal relative to a transgenic non-human animal of step (a) which has not been administered the agent.

The agent may be useful in the treatment and prophylaxis of conditions such as cancer as discussed herein. The agents may also be incorporated in a pharmaceutical composition as described herein.

The activity of the proteins, substances, compounds, antibodies, nucleic acid molecules, agents, and compositions of the invention may be confirmed in animal experimental model systems. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating the ED_{50} (the dose therapeutically effective in 50% of the population) or LD_{50} (the dose lethal to 50% of the population) statistics. The therapeutic index is the dose ratio of therapeutic to toxic effects and it can be expressed as the ED_{50}/LD_{50} ratio. Pharmaceutical compositions which exhibit large therapeutic indices are preferred.

The following non-limiting examples are illustrative of the present invention:

Examples

Example 1

MATERIALS AND METHODS

Identification of positive PAC and BAC genomic clones from a human genomic DNA library

The sequence of PSA, KLK1, KLK2, NES1 and Zyme genes is already known. Polymerase chain reaction (PCR)-based amplification protocols have been developed which allowed generation of PCR products specific for each one of these genes. Using these PCR products as probes, labeled with ^{32}P, a human genomic DNA PAC library and a human genomic DNA BAC library was screened for the purpose of identifying positive clones of approximately 100-150 Kb long. The general strategies for these experiments have been published elsewhere (14). The genomic libraries were spotted in duplicate on nylon membranes and positive clones were further confirmed by Southern blot analysis as described (14).

DNA sequences on chromosome 19

The Lawrence Livermore National Laboratory participates in the sequencing of the human genome project and focuses on sequencing chromosome 19. Large sequencing information on this chromosome is available at the website of the Lawrence Livermore National Laboratory (http://www-bio.llnl.gov/genome/gemnome.html).

Approximately 300 Kb of genomic sequences were obtained from that website, encompassing a region on chromosome 19q13.3 - 13.4, where the known kallikrein genes are localized. This 300 Kb of sequence is represented by 8 contigs of variable lengths. By using a number of different computer

programs, an almost contiguous sequence of the region was established as shown diagramatically in Figure 1 and Figure 28. Some of the contigs were reversed as shown in Figure 1 in order to reconstruct the area on both strands of DNA.

By using the published sequences of PSA, KLK2, NES1 and Zyme and the computer software BLAST 2, using alignment strategies, the relative positions of these genes on the contiguous map were identified (Figure 28). These known genes served as hallmarks for further studies. An EcoR1 restriction map of the area is also available at the website of the Lawrence Livermore National Laboratory. Using this restriction map and the computer program WebCutter (http://www.firstmarket.com/cutter/cut2.html), a restriction study analysis of the available sequence was performed to further confirm the assignment and relative positions of these contigs along chromosome 19. The obtained configuration and the relative location of the known genes are presented in Figure 1.

Gene prediction analysis

For exon prediction analysis of the whole genomic area, a number of different computer programs were used. All the programs were initially tested using known genomic sequences of the PSA, Zyme, and NES1 genes. The more reliable computer programs, GeneBuilder (gene prediction), GeneBuilder (exon prediction), Grail 2 and GENEID-3 were selected for further use.

Protein homology searching

Putative exons of the new genes were first translated to the corresponding amino acid sequences. BLAST homology searching for the proteins encoded by the exons of the putative new genes were performed using the BLASTP program and the Genbank databases.

RESULTS

Relative position of PSA, KLK2, Zyme and NES1 on Chromosome 19

Screening of the human BAC library identified two clones which were positive for the Zyme gene (clones BAC 288H1 and BAC 76F7). These BACs were further analyzed by PCR and primers specific for PSA, NES1, KLK1 and KLK2. These analyses indicated that both BACs were positive for Zyme, PSA and KLK2 and negative for KLK1 and NES1 genes.

Screening of the human PAC genomic library identified a PAC clone which was positive for NES1 (clone PAC 34B1). Further PCR analysis indicated that this PAC clone was positive for NES1 and KLK1 genes and negative for PSA, KLK2 and Zyme. Combination of this information with the EcoR1 restriction map of the region allowed establishment of the relative positions of these four genes. PSA is the most centromeric, followed by KLK2, Zyme and NES1. Further alignment of the known sequences of these genes with the 300 Kb contig enabled precise localization of all four genes and determination of the direction of transcription, as shown by the arrows in Figure 1. The KLK1 gene sequence was not identified on any of these contigs and appears to be further telomeric to NES1 (since it is co-localized on the same PAC as NES1).

Identification of new genes

A set of rules was used to consider the presence of a new gene in the genomic area of interest as follows:

1. Clusters of at least 3 exons should be found.
2. Only exons with high prediction score ("good" or "excellent" quality, as indicated by the searching programs) were considered for the construction of the putative new genes.
3. Exons predicted were reliable only if they were identified by at least two different exon prediction programs.

By using this strategy, eleven putative new genes were identified of which three were found on subsequent homology analysis to be known genes not previously mapped i.e. the human stratum corneum chymotrypsin enzyme (HSCCE), human neuropsin, and trypsin-like serine protease (TLSP). Their relative location is shown in Figure 1. The five genes all have variable homologies with known human or animal kallikrein proteins and/or other known serine proteases (depicted as KLK-L1, KLK-L2, KLK-L3, KLK-L4 and KLK-L5 in Figure 1 and KLK-L1 to KLK-L6 in Figure 28).

In Tables 1 to 5, the preliminary exon structure and partial protein sequence for each one of the newly identified genes is shown. In Table 6, some proteins are presented which appear, on preliminary analysis, to be homologous to the proteins encoded by the putative new genes. SEQ. ID. NOs. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, and 67 show amino acid sequences of KLK-L1-KLK-L6, and SEQ. ID. NOs.1, 13, 21, 43, 56, and 65 show nucleic acid sequences of the genes encoding KLK-L1- KLK-L6.

DISCUSSION

Prediction of protein-coding genes in newly sequenced DNA becomes very important after the establishment of large genome sequencing projects. This problem is complicated due to the exon-intron structure of the eukaryotic genes which interrupts the coding sequence in many unequal parts. In order to predict the protein-coding exons and overall gene structure, a number of computer programs were developed. All these programs are based on the combination of potential functional signals with the global statistical properties of known protein-coding regions (15). However, the most powerful approach for gene structure prediction is to combine information about potential functional signals (splice sites, translation start or stop signal etc.) together with the statistical properties of coding sequences (coding potential) along with information about homologies between the predicted protein and known protein families (16).

In mouse and rat, kallikreins are encoded by large multigene families and these genes tend to cluster in groups with a distance as small as 3.3 – 7.0 Kb (3). A strong conservation of gene order between human chromosome 19q13.1 – q13.4 and 17 loci in a 20-cM proximal part of mouse chromosome 7, including the kallikrein locus, has been documented (17).

In humans, only a few kallikrein genes were identified. In fact, only KLK1, KLK2 and KLK3 (PSA) are considered to represent the human kallikrein gene family (9). The work described herein provides strong evidence that a large number of kallikrein-like genes are clustered within a 300Kb region around chromosome 19q13.2 – q13.4. The three established human kallikreins (KLK1, KLK2, KLK3), Zyme and NES1, as well as the stratum corneum chymotrypticn enzyme, neuropsin, and TLSP (trypsin-like serine protease) and another five new genes , KLK-L1 to KLK-L5, may constitute a large gene family. This will bring the total number of kallikrein or kallikrein-like genes in this region of chromosome 19 to thirteen.

The human stratum corneum chymotryptic enzyme (19), neuropsin (20) and trypsin-like serine

protease (TLSP) (21) are three previously characterized genes which have many structural similarities with the kallikreins and other members of the serine protease family. However, they have not been mapped in the past. Their precise mapping in the region of the kallikrein gene family indicates that these three genes, along with the ones that were newly identified, or are already known, constitute a family that likely originated by duplication of an ancestral gene. The relative localization of all these genes is depicted in Figure 1.

Kallikrein genes are a subfamily of serine proteases, traditionally characterized by their ability to liberate lysyl-bradykinin (kallidin) from kininogen (18). More recently, however, a new, structural concept has emerged to describe kallikreins. From accumulated sequence data, it is now clear that the mouse has many genes with high homology to kallikrein coding sequences (19-20). Richard and co-workers have contributed to the concept of a " kallikrein multigene family" to refer to these genes (21-22). This definition is not based much on specific enzymatic function of the gene product, but more on its sequence homology and their close linkage on mouse chromosome 7. In humans, only KLK1 meets the functional definition of a kallikrein. KLK2 has trypsin-like enzymatic activity and KLK3 (PSA) has very weak chymotrypsin-like enzymatic activity. These activities of KLK2 and KLK3 are not known to liberate biologically active peptides from precursors. Based on the newer definition, members of the kallikrein family include, not only the gene for the kallikrein enzyme, but also genes encoding other homologous proteases, including the enzyme that processes the precursors of the nerve growth factor and epidermal growth factor (8). Therefore, it is important to note the clear distinction between the enzyme kallikrein and a kallikrein or a kallikrein-like gene.

In carrying out the study only exons were considered which were predicted with "good" or "excellent" quality and only exons were considered which were predicted by at least two different programs. Moreover, the presence of a putative gene was only considered when at least three exons clustered coordinately in that region. Additional evidence that these new genes are indeed homologous to the known kallikreins and other serine proteases comes from comparison of the intron phases. As published previously (14), trypsinogen, PSA and NES1 have 5 coding exons of which the first has intron phase I (the intron occurs after the first nucleotide of the codon), the second has intron phase II (the intron occurs after the second nucleotide and the codon), the third has intron phase I and the fourth has intron phase 0 (the intron occurs between codons). The fifth exon contains the stop codon. The intron phases of the predicted new kallikrein-like genes follow these rules and are shown in the respective tables. Further support comes from the identification in the new genes, of the conserved amino acids of the catalytic domain of the serine proteases, as presented in Tables 1 - 5.

In order to test the accuracy of the computer programs, known genomic areas containing the PSA, Zyme and KLK2 genes were tested. Two of these programs (Grail 2 and GeneBuilder) were able to detect about 95% of the tested known genes. Matches with expressed sequence tag sequences (EST) can also be employed for gene structure prediction in the GeneBuilder program and this can significantly improve the power of the program especially at high stringency (e.g. >95% homology).

In mouse, ten of the kallikrein genes appear to be pseudogenes (9).

Example 2

PROSTASE/KLK-L1 in prostate and breast tissues

The fine mapping of the prostase/KLK-L1 gene and its chromosomal localization in relation to a number of other homologous genes also mapping to the same region are described. In addition, extensive tissue expression studies were carried out that demonstrate that, in addition to prostate (which shows the highest expression), that prostase/KLK-L1 is also expressed in female breasts, testis, adrenals, uterus, colon, thyroid, brain, spinal cord and salivary glands. Furthermore, the gene is up-regulated by androgens and progestins in the breast carcinoma cell line BT-474.

Materials and Methods

DNA sequences on chromosome 19

Large DNA sequencing data for chromosome 19 is available at the web site of the Lawrence Livermore National Laboratory (LLNL) (http://www-bio.llnl. gov/genome /genome.html). Approximately 300 Kb of genomic sequence was obtained from that web site, encompassing a region on chromosome 19q13.3 - 13.4, where the known kallikrein genes are localized. This sequence is represented by 9 contigs of variable lengths. By using the sequences of PSA, KLK2, NES1 and protease M and the alignment program BLAST 2 (37), the relative positions of these genes on the contiguous map were located.

Gene prediction analysis

For exon prediction analysis of the whole genomic area, a number of different computer programs were used. Originally all these programs were tested using the known genomic sequences of the PSA, protease M and NES1 genes. The most reliable computer programs GeneBuilder (gene prediction)[http://125.itba.mi.cnr.it/~webgene/genebuilder.html] GeneBuilder (exon prediction) [http://125.itba.mi.cnr.it/~webgene/genebuilder.html] , Grail 2 [http://compbio.ornl.gov], and GENEID-3 [http://apolo.imim.es/geneid.htmll] were selected for further use.

Protein homology searching

Putative exons of the newly identified gene were first translated to the corresponding amino acid sequences. BLAST homology searching for the proteins encoded by the exons were performed using the BLASTP program and the GenBank databases (37).

Searching expressed sequence tags (ESTs)

Sequence homology searching was performed using the BLASTN alogrithm (37) on the National Center for Biotechnology Information web server (http://www ncbi.nlm.nih.gov/BLAST/) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. (38) consortium through Research Genetics Inc, Huntsville, AL and from The Institute for Genomic Research (TIGR) (http://WWW.TIGR.ORG/ tdb/tdb.html) (Table 7). Clones were propagated, purified and then sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Breast cancer cell line and stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. BT-474 cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%),

antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the breast cancer cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 μg of total RNA was reverse transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 μl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (Table 8), PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

Tissue expression of KLK-L1

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described above for the tissue culture experiments and used for PCR reactions with the primers described in Table 8 (SEQ. ID. Nos 5-12). Tissue cDNAs were amplified at various dilutions.

Cloning and sequencing of the PCR products

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, by an automated DNA sequencer.

Results

Identification of the prostase/KLK-L1 gene

The exon prediction strategy of the 300Kb DNA sequences around chromosome 19q13.3 - q13.4 identified a novel gene with a structure reminiscent of a serine protease. The major features of this gene were its homology, at the amino acid and DNA level, with other human kallikrein genes; the conservation of the catalytic triad (histidine, aspartic acid, and serine), the number of exons and the complete conservation of the intron phases.

EST sequence homology search

EST sequence homology search of the putative exons obtained from the gene prediction programs (as described above) against the human EST database (dbEST) revealed five expressed sequence tags

(ESTs) with >95 % identity to the putative exons of the gene (Table 7). Positive clones were obtained and the inserts were sequenced from both directions. Alignment was used to compare between the EST sequences and the exons predicted by the computer programs, and final selection of the exon-intron splice sites was made according to the EST sequences. Furthermore, many of the ESTs were overlapping, further ensuring the accuracy of the data.

The coding sequence of the klk-L2 gene is shown in SEQ. ID. NO. 1 and GenBAnk Accession # AF135023. The exons of the gene are as follows: exon 1 (939-999); exon 2 (2263-2425); exon 3 (2847-3097); exon 4 (3181-3317); and exon 5 (4588-4740). The amino acid sequence of KLK-L2 proteins are shown in SEQ. ID. Nos. 2 and 3.

Mapping and chromosomal localization of prostase /KLK-L1 gene

Alignment of the prostase/ KLK-L1 sequence and the sequences of other known kallikrein genes within the 300 Kb area of the contigs constructed at the Lawrence Livermore National Laboratory enabled precise localization of all genes and to determine the direction of transcription, as shown in Figure 2. The distance between PSA and KLK2 genes was calculated to be 12,508 bp. The prostase/KLK-L1 gene is 26,229 bp more telomeric to KLK2 and transcribes in the opposite direction. The zyme gene is about 51 Kb more telomeric to the prostase gene and transcribes in the same direction. The human stratum corneum chymotryptic enzyme gene, the neuropsin gene and the NES 1 gene are all further telomeric to zyme and all transcribe in the same direction as zyme.

Tissue expression of the prostase/KLK-L1 gene

The tissues that express the prostase/KLK-L1 gene were assessed by RT-PCR. The experiments were performed at various dilutions of the cDNAs to obtain some information about the relative levels of expression. RT-PCR for actin was used as a positive control and RT-PCR for the PSA cDNA was used as another positive control with tissue restricted specificity. Positive ESTs for prostase/KLK-L1 were used as controls for the PCR procedure. The PSA gene was found to be highly expressed in the prostate, as expected, and to a lower extent in mammary and salivary glands as also expected from recent literature reports (24, 25). Very low expression of PSA in the thyroid gland, trachea and testis was also found, a finding that accords with recent RT-PCR data by others (26).

The tissue expression of prostase/KLK-L1 is summarized in Table 9 and Figure 3. This protease is primarily expressed in the prostate, testis, adrenals, uterus, thyroid, colon, central nervous system and mammary tissues, and, at much lower levels in other tissues. The specificity of the RT-PCR procedure was verified for prostase/KLK-L1 by cloning the PCR products from mammary, testicular and prostate tissues and sequencing them. One example with mammary tissue is shown in Figure 4. All cloned PCR products were identical in sequence to the cDNA sequence reported for the prostase/KLK-L1.

Hormonal regulation of the prostase/KLK-L1 gene

The steroid hormone receptor-positive breast carcinoma cell line BT-474 was used as a model system to evaluate whether prostase/KLK-L1 expression is under steroid hormone regulation. As shown in Figure 5, the controls worked as expected i. e., actin positivity without hormonal regulation in all cDNAs, only estrogen up-regulation of the pS2 gene and up-regulation of the PSA gene by androgens and

progestins. Prostase/KLK-L1 is up-regulated primarily by androgens and progestins, similarly to PSA. This up-regulation was dose-dependent and it was evident at steroid hormone levels $\geq 10^{-10}$ M.

DISCUSSION

The KLK3 gene encodes for PSA, a protein that currently represents the best tumor marker available (24). Since in rodents there are so many kallikrein genes, the restriction of this family to only 3 genes in humans was somewhat surprising. More recently, new candidate kallikrein genes in humans have been discovered, including NES1 (13) and zyme/protease M/neurosin (10-12). The known kallikreins and the newly discovered kallikrein-like genes share the following similarities: (a) they encode serine proteases (b) they have five coding exons (c) they share significant DNA and protein homologies with each other (d) they map in the same locus on chromosome 19q13.3-q13.4, a region that is structurally similar to an area on mouse chromosome 7, where all the mouse kallikrein genes are localized (e) they appear to be regulated by steroid hormones. Prostase/KLK-L1 is a member of the same family since these common characteristics are also shared by the newly discovered gene.

The exact localization of the KLK-L1 gene and its position in relation to other genes in the area (Figure 2) was determined. Prostase/KLK-L1 lies between KLK2 and zyme.

Irwin et al. (27) have proposed that the serine protease genes can be classified into five different groups according to intron position. The established kallikreins (KLK1, KLK2, and PSA), trypsinogen and chymotrypsinogen belong to a group that has: (1) an intron just downstream from the codon for the active site histidine residue, (2) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (3) a third intron just upstream from the exon containing the codon for the active site serine residue. As seen in Figure 6, the genomic organization of prostase/KLK-L1 gene is very similar to this group of genes. The lengths of the coding parts of exons 1-5 are 61,163, 263, 137 and 153 bp, respectively, which are close or identical to the lengths of the exons of the kallikrein genes and also, similar or identical to those of other newly discovered genes in the same chromosomal region like the NES1(14), zyme/protease M/neurosin (10-12) and neuoropsin (28) genes.

The sensitive RT-PCR protocol reveals that the KLK-L1 enzyme is expressed in prostatic tissue and it is also expressed in significant amounts in other tissues, including testis, female mammary gland, adrenals, uterus, thyroid, colon, brain, lung and salivary glands (Figure 3 and Table 9). The specificity of the RT-PCR primers was verified by sequencing the obtained PCR products, with one example shown in Figure 4 (SEQ.ID.NO. 4). Tissue culture studies with the breast carcinoma cell line BT-474 further confirm not only the ability of these cells to produce prostase/KLK-L1 but also its hormonal regulation (Figure 5).

An interesting theme is now developing involving the group of homologous genes on chromosome 19q13.3(PSA, KLK2, prostase, zyme, and NES1). The combined data suggest that all of them are expressed in prostate and breast tissues, and all of them are hormonally regulated. All these genes may be part of a cascade pathway that plays a role in cell proliferation, differentiation or apoptosis by regulating (positively or negatively) growth factors or their receptors or cytokines, through proteolysis (30). Also interesting is the linkage of locus 19q13 to solid tumors and gliomas (31) which raises the possibility that some of the genes in the region may be disrupted by rearrangements.

The KLK-1L gene encodes for a serine protease that shows homology with other members of the kallikrein gene family and maps to the same chromosomal location. Many structural features of the kallikreins are conserved in prostase/KLK-L1. The precise mapping of this gene between the two known genes KLK2 and zyme is presented. It is further demonstrated that prostase/KLK-L1 is expressed in many tissues, in addition to the prostate, including the female breast. This gene is also herein referred to as 'prostase'. It has been further demonstrated, using breast carcinoma cell lines, that prostase/KLK-L1 can be produced by these cells and that its expression is significantly up-regulated by androgens and progestins. Based on information for other homologous genes in the area (PSA, zyme, and NES1), prostase/KLK-L1 may be involved in the pathogenesis and/or progression of prostate, breast and possibly other cancers.

Example 3

IDENTIFICATION OF THE KLK-L2 GENE

Materials and Methods

DNA sequence on chromosome 19

Sequencing data of approximately 300Kb of nucleotides on chromosome 19q13.3-q13.4 was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (http://www-bio.llnl.gov/genome/genome.html). This sequence was in the form of 9 contigs of different lengths. A restriction analysis study of the available sequences was performed using the "WebCutter" computer program (http://www.firstmarket. com/cutter/cut2.html) and with the aid of the EcoR1 restriction map of this area (also available from the LLNL web site) an almost contiguous stretch of genomic sequences was constructed. The relative positions of the known kallikrein genes: PSA (GenBank accession # X14810), KLK2 (GenBank accession # M18157), and zyme (GenBank accession # U60801) was determined using the alignment program BLAST 2 (37).

New Gene Identification

A number of computer programs were used to predict the presence of putative new genes in the genomic area of interest. These programs were initially tested using the known genomic sequences of the PSA, protease M and NES1 genes. The most reliable computer programs GeneBuilder (gene prediction) (http://l25.itba.mi.cnr.it/~webgene/genebuilder.html) GeneBuilder (exon prediction) (http://l25.itba.mi.cnr.it/~webgene/genebuilder.html), Grail 2 (http://compbio.ornl.gov) and GENEID-3 (http://apolo.imim.es/geneid.html) were selected for further use.

Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (http://www ncbi.nlm.nih.gov/BLAST/) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL (Table 10). The clones were propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Rapid amplification of cDNA ends (5' RACE)

According to the EST sequence data and the predicted structure of the gene, two gene-specific primers were designed (R1 & R2) (Table 11). Two rounds of RACE reactions (nested PCR) were performed with 5μl Marathon Ready™ cDNA of human testis (Clontech, Palo Alto, CA, USA) as a template. The reaction mix and PCR conditions were conducted according to the manufacturer's recommendations. In brief, denaturation was done for 5 min at 94°C followed by 94° C for 5 sec followed by 72°C for 2 min for 5 cycles, then 94°C for 5 sec followed by 70° C for 2 min for 5 cycles then 94°C for 5 sec followed by 65°C for 2 min for 30 cycles for the first reaction and 25 cycles for the nested PCR reaction.

Tissue expression

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described below for the tissue culture experiments and used for PCR reactions with the primers described in Table 11 (SEQ. ID. Nos 9-12, 15-20). Tissue cDNAs were amplified at various dilutions.

Breast cancer cell line and hormonal stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the breast cancer cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 μg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 μl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (Table 11) and PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Structure analysis

Multiple alignment was performed using the Clustal X software package available at: ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/ (clustalx1.64b.msw.exe) and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA (kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html). Phylogenetic studies were performed using the Phylip software package available at: http://evolution.genetics. washington.edu/phylip/getme.html. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html). Signal peptide was predicted using the "SignalP" server (http://www.cbs.dtu.dk/services/ signal). Protein structure analysis was performed by "SAPS" (structural analysis of protein sequence) program (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html).

RESULTS

Computer analysis of the genomic sequence predicted a putative new gene consisting of four exons. This gene was detected by all programs used and all exons had high prediction scores. EST sequence homology search of the putative exons against the human EST database (dbEST) revealed nine expressed sequence tag (EST) clones from different tissues with >95 % identity to the putative exons of the gene (Table 10). Positive clones were obtained and the inserts were sequenced from both directions. The "Blast 2 sequences" program was used to compare the EST sequences with the predicted exons, and final selection of the exon-intron splice sites was done according to the EST sequences. The presence of many areas of overlap between the various EST sequences allowed further verification of the structure of the new gene. The coding sequence of the gene is shown in SEQ. ID. NO. 13 and GenBank Accession #AF135028. The 3' end of the gene was verified by the presence of poly A stretches that are not present in the genomic sequence at the end of two of the sequenced ESTs. One of the sequenced ESTs revealed the presence of an additional exon at the 5'end. The nucleotide sequence of this exon matches exactly with the genomic sequence. To further identify the 5' end of the gene, 5' RACE was performed but no additional sequence could be obtained. However, as is the case with other kallikreins, the presence of further up-stream untranslated exon(s) could not be excluded. The amino acid sequence of KLK-L2 is shown in SEQ. ID. No. 14.

Mapping and chromosomal localization of the KLK-L2 gene

Alignment of KLK-L2 gene and the sequences of other known kallikrein genes within the 300 Kb area of interest enabled precise localization of all genes and determination of the direction of transcription, as shown by the arrows in Figure 8. The PSA gene was found to be the most centromeric, separated by 12,508 base pairs (bp) from KLK2, and both genes are transcribed in the same direction (centromere to telomere). The prostase/KLK-L1 gene is 26,229 bp more telomeric and transcribes in the opposite

direction, followed by KLK-L2. The distance between KLK-L1 and KLK-L2 is about 35 Kilobases (Kb). The zyme gene is 5,981 bp more telomeric and the latter 3 genes are all transcribed in the same direction (Figure 8).

Structural characterization of the KLK-L2 gene and its protein product

The KLK-L2 gene, as presented in Figure 7, is formed of 5 coding exons and 4 intervening introns, spanning an area of 9,349 bp of genomic sequence on chromosome 19q13.3-q13.4. The lengths of the exons are 73, 262, 257,134, and 156 bp, respectively. The intron/exon splice sites (mGT....AGm) and their flanking sequences are closely related to the consensus splicing sites (-mGTAAGT ...CAGm-) (32). The presumptive protein coding region of the KLK-L2 gene is formed of 879 bp nucleotide sequence encoding a deduced 293-amino acid polypeptide with a predicted molecular weight of 32 KDa. There are two potential translation initiation codons (ATG) at positions 1 and 25 of the predicted first exon (numbers refer to SEQ. ID. NO . 13 and GenBank Accession #AF135028). It is assumed that the first ATG will be the initiation codon, since : (1) the flanking sequence of that codon (GCGGCCATGG) matches closely with the Kozak consensus sequence for initiation of translation (GCC A/G CCATGG) (33) and is exactly the same as that of the homologous zyme gene. At this initiation codon, the putative signal sequence at the N-terminus is similar to other trypsin-like serine proteases (prostase and EMSP) (Figure 9). The cDNA ends with a 328 bp of 3' untranslated region containing a conserved poly adenylation signal (AATAAA) located 11 bp up-stream of the poly A tail (at a position exactly the same as that of the zyme poly A tail)(11).

A hydrophobicity study of the KLK-L2 gene shows a hydrophobic region in the N-terminal region of the protein (Figure 10), suggesting that a presumed signal peptide is present. By computer analysis, a 29-amino acid signal peptide is predicted with a cleavage site at the carboxyl end of Ala^{29}. For better characterization of the predicted structural motif of the KLK-L2 protein, it was aligned with other members of the kallikrein multi-gene family, (Figure 9), and the predicted signal peptide cleavage site was found to match with the predicted signal cleavage sites of zyme (11), KLK1(1), KLK2 (8), and KLK-L1. Also, sequence alignment supports, by analogy, the presence of a cleavage site at the carboxyl end of Ser^{66}, which is the exact site predicted for cleavage of the activation peptide of all the other kallikreins aligned in Figure 9. Interestingly, the starting amino acid sequence of the mature protein (I I N G (S) D C) is conserved in the prostase and enamel matrix serine proteinase 1 (EMSP) genes. Thus, like other kallikreins, KLK-L2 is likely also synthesized as a preproenzyme that contains an N-terminal signal peptide (prezymogen) followed by an activation peptide and the enzymatic domain.

The presence of aspartate (D) in position 239 suggests that KLK-L2 will possess a trypsin-like cleavage pattern like most of the other kallikreins (e.g., KLK1, KLK2, TLSP, neuropsin, zyme, prostase, and EMSP) but different from PSA which has a serine (S) residue in the corresponding position, and is known to have a chymotrypsin like activity (Figure 9). The dotted region in Figure 9 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L2 or other members of the kallikrein-like gene family (11).

Homology with the kallikrein multi-gene family

The mature 227-amino acid sequence of the predicted protein was aligned against the GenBank

database and the known kallikreins using the "BLASTP" and "BLAST 2 sequence" programs. KLK-L2 is found to have 54% amino acid sequence identity and 68% similarity with the enamel matrix serine proteinase 1 (EMSP1) gene, 50% identity with both trypsin like serine protease (TLSP) and neuropsin genes and 47%, 46%, and 42% identity with trypsinogen, zyme, and PSA genes, respectively. The multiple alignment study shows that the typical catalytic triad of serine proteases is conserved in the KLK-L2 gene (H^{108}, D^{153}, and S^{245}) and, as the case with all other kallikreins, a well conserved peptide motif is found around the amino acid residues of the catalytic triad [i.e., histidine (WLLTAA*H*C), serine(GD*S*GGP), and aspartate(*D*LMLI)] (10, 11).

Twelve cysteine residues are present in the putative mature KLK-L2 protein, ten of them are conserved in all the serine proteases that are aligned in Figure 9, and would be expected to form disulphide bridges. The other two cysteines (C^{178} and C^{279}) are not found in PSA, KLK1, KLK2 or trypsinogen, however, they are found in similar positions in prostase, EMSP1, zyme, neuropsin, and TLSP genes and are expected to form an additional disulphide bond. Twenty nine "invariant" amino acids surrounding the active site of serine proteases have been described (39). Of these, twenty-six are conserved in KLK-L2. One of the non-conserved amino acids (Ser^{210} instead of Pro) is also found in prostase and EMSP1 genes, the second (Leu^{103} instead of Val) is also found in TLSP gene, and the third (Val^{174} instead of Leu) is also not conserved in prostase or EMSP1 genes. According to protein evolution studies, each of these amino acid changes represents a conserved evolutionary substitution to a protein of the same group (39).

Evolution of the KLK-L2 gene

To predict the phylogenetic relatedness of the KLK-L2 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA method (Figure 10). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped the KLK-L2 with KLK-L1, EMSP1, and TLSP (40, 41).

Tissue expression of the KLK-L2 gene

As shown in Table 12 and Figure 11, the KLK-L2 gene is primarily expressed in the brain, mammary gland, and testis but lower levels of expression are found in many other tissues. In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced.

Hormonal regulation of the KLK-L2 gene

A steroid hormone receptor positive breast cancer cell line (BT-474) was used as a model to verify whether the KLK-L2 gene is under steroid hormone regulation. PSA was used as a control known to be upregulated by androgens and progestins and pS2 as an estrogen upregulated control. The results indicate that KLK-L2 is up-regulated by estrogens and progestins (Figure 12).

Expression of KLK-L2 in Ovarian Tissues

KLK-L2 is up-regulated (overexpressed) in ovarian tumors (Figure 13).

Discussion

With the aid of computer programs for gene prediction and the available EST database, a new gene, named KLK-L2 (for kallikrein like gene 2) was identified. The 3' end of the gene was verified by the

presence of "poly A" stretches in the sequenced ESTs which were not found in the genomic sequence, and the start of translation was identified by the presence of a start codon in a well conserved consensus Kozak sequence.

As is the case with other kallikreins, the KLK-L2 gene is composed of 5 coding exons and 4 intervening introns and, except for the second coding exon, the exon lengths are comparable to those of other members of the kallikrein gene family (Figure 6). The exon-intron splice junctions were identified by comparing the genomic sequence with the EST sequence and were further confirmed by the conservation of the consensus splice sequence (-mGT......AGm-) (32), and the fully conserved intron phases, as shown in Figure 6. Furthermore, the position of the catalytic triad residues in relation to the different exons is also conserved (Figure 6). As is the case with most other kallikreins, except PSA and HSCCE, KLK-L2 is more functionally related to trypsin than to chymotrypsin (34). The wide range of tissue expression of KLK-L2 should not be surprising since, by using the more sensitive RT-PCR technique instead of Northern blot analysis, many kallikrein genes were found to be expressed in a wide variety of tissues including salivary gland, kidney, pancreas, brain, and tissues of the reproductive system (uterus, mammary gland, ovary, and testis) (34). KLK-L2 is highly expressed in the brain. Another kallikrein, neuropsin, was also found to be highly expressed in the brain and has been shown to have important roles in neural plasticity in mice (35). Also, the zyme gene is highly expressed in the brain and appears to have amyloidogenic potential (11). Taken together, these data point to a possible role of KLK-L2 in the central nervous system.

It was initially thought that each kallikrein enzyme has one specific physiological substrate. However, the increasing number of substrates, which purified proteins can cleave *in vitro*, has led to the suggestion that they may perform a variety of functions in different tissues or physiological circumstances. Serine proteases encode protein cleaving enzymes that are involved in digestion, tissue remodeling, blood clotting etc., and many of the kallikrein genes are synthesized as precursor proteins that must be activated by cleavage of the propeptide. The predicted trypsin-like cleavage specificity of KLK-L2 makes it a candidate activator of other kallikreins or it may be involved in a "cascade" of enzymatic reactions similar to those found in fibrinolysis and blood clotting (36).

In conclusion, a new member of the human kallikrein gene family, KLK-L2 was characterized. This gene is hormonally regulated and it is mostly expressed in the brain, mammary gland and testis. KLK-L2 may be useful as a tumor marker.

Example 4

Materials and methods

Strategy for new gene discovery

Sequencing data of approximately 300 kb, around chromosome 19q13.3-q13.4, was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (http://www-bio.llnl.gov/ genome/ genome. html). Different computer programs were used for putative new gene prediction, as previously described.

RT-PCR for KLK-L3 cDNA

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA.

cDNA was prepared as described below and used for PCR amplification. A primer set (L3-F1 and L3-R1) was used to identify the presence of the gene in tissues, and the reverse primer (L3-R1) was used with another primer (L3-F2) to amplify and clone the full cDNA of the gene. These primer sequences are shown in Table 13 (SEQ. ID. Nos. 9-12, 24-26). Tissue cDNAs were amplified at various dilutions.

Reverse transcriptase polymerase chain reaction.

2 μg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL, Gaithersburg, MD). The final volume was 20 μl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequence, two gene-specific primers (L3-F1 and L3-R1) were designed (Table 13, SEQ. ID. Nos. 9-12, 24-26) and PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute, and a final extension at 63°C for 10 minutes. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

Breast cancer cell line and hormonal stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for total RNA extraction by the Trizol method (Gibco-BRL). cDNA was prepared and amplified as described above. Control genes (PSA, pS2, and actin) were amplified as previously described herein.

Cloning and sequencing of the PCR products.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Identification of positive PAC and BAC genomic clones from human genomic DNA libraries

The PCR product generated with primer set Z1S and Z1AS (Table 14, SEQ.ID.NOS. 27-42), was purified and then labeled with ^{32}P by the random primer method (Sambrook, supra) and used as a probe to screen a human genomic DNA BAC library, spotted in duplicate on nylon membranes, for identification of positive clones. The filters were hybridized in 15% formamide, 500 mM Na_2HPO_4, 7% SDS, 1% BSA (w/v) at 65°C overnight, then washed sequentially with 2X SSC, 1X SSC, 0.2X SSC, containing 0.1% SDS

at 65°C, and then exposed to X-ray film as described (Sambrook, supra). Positive clones were obtained, plated on selective LB medium, and then a single colony was transferred into LB broth for overnight cultures. A PAC clone positive for NES1 was identified by a similar methodology as described elsewhere (14). PAC and BAC libraries were constructed by de Jong and associates (42). Purification of BAC and PAC DNA was done by a rapid alkaline lysis miniprep method, which is a modification of the standard Qiagen-Tip method. Positive clones were further confirmed by Southern blot analysis as described (Sambrook, supra).

5' Rapid amplification of cDNA ends (5' RACE)

According to the EST sequences and the computer-predicted structure of the KLK-L3 gene, two gene specific primers were designed. Two rounds of RACE reactions (nested PCR) were performed with 5μl Marathon Ready ™ cDNA of human testis (Clontech) as a template. The reaction mix and PCR conditions were selected according to the manufacturer's recommendations. Positive bands were gel-purified using Qiagen Gel Purification kits according to manufacturer's recommendations.

Gene-specific amplification of other genes from genomic DNA

According to the published sequence of prostatic specific antigen (PSA), human renal kallikrein (KLK1), human glandular kallikrein (KLK2), normal epithelial cell-specific 1gene (NES1), KLK-L1, KLK-L2 and zyme genes, gene-specific primers were designed for each of these genes (Table 14) and developed polymerase chain reaction (PCR)-based amplification protocols which allowed us to generate specific PCR products with genomic DNA as a template. The PCR reactions were carried out as described above but by using an annealing/extension temperature of 65°C.

Structure analysis studies.

Multiple alignment was performed using the clustal X software package available at: ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/(clustalx1.64b.msw.exe) and the multiple alignment program available from the Baylor College of Medicine (BCM) search launcher (kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html). Phylogenetic studies were performed using the Phylip software package available from: (http://evolution.genetics.washington.edu/phylip/getme.html). Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Prompters" program. Hydrophobicity study was performed using the BCM search launcher programs (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html). Signal peptide was predicted using the SignalP WWW server (http://www.cbs.dtu.dk/services/ signal). Protein structure analysis was performed by SAPS (structural analysis of protein sequence) program (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html).

Results:

Construction of a contiguous map of the human kallikrein locus on chromosome 19q13.3-q13.4

Sequence information around the human chromosome 19q13.3-q13.4 locus (the proposed kallikrein locus) is available at the Lawrence Livermore National Laboratory web site. Sequences of approximately 300 kb in length were obtained. These sequences were in the form of contigs of different lengths. A restriction analysis study of the contigs was performed using various computer programs. With

the aid of the EcoR1 restriction map of this area which is also available at the LLNL web site, the relative positions of these contigs was defined in relation to each other. Some contigs were overlapping, enabling construction of a contiguous segment; however, three gaps were present. <BLAST> analysis of these segments against the GenBank database (37) enabled the precise location of two classical kallikreins, namely PSA and KLK2 to be defined. Other newly discovered serine proteases were localized which are homologous with the kallikrein genes, namely protease M/zyme/neurosin (10, 11, 12), human stratum corneum chymotryptic enzyme (HSSCE) (55), neuropsin (28), normal epithelial cell-specific 1 gene (NES1) (13), trypsin-like serine protease (TLSP) (GenBank accession # AF164623), KLK-L1 (SEQ.ID.NO. 1) and KLK-L2 (SEQ.ID.NO. 13). The gaps in the 300 kb genomic sequence were partially filled as follows:

(a) The margins of the first gap were found to contain the 5' and 3' ends of the KLK2 gene; this gap was filled with the genomic structure of the KLK2 gene (GenBank Accession # M18157).

(b) The margins of the third gap (gaps are numbered from centromere to telomere) were found to have the 3' and 5' ends of the zyme gene mRNA sequence; thus, a radiolabeled probe specific for the zyme gene was used to screen a human BAC library and two positive clones were obtained. Restriction analysis was performed, followed by Southern blotting and a fragment containing the zyme gene was obtained and sequenced, thus filling this gap.

(c) The second gap (between KLK-L1 and KLK-L2 genes) still exists and the EcoR1 restriction map of this area was used to approximately define its length (Figure 14).

Further support for the relative locations of these genes was obtained by performing PCR reactions with gene-specific primers to screen genomic DNA clones. The most centromeric group of genes (PSA, KLK2, KLK-L1, KLK-L2 and zyme) were found to be clustered in one genomic BAC clone, and the next group (HSCCE, neuropsin,KLK-L3 and NES1) were found to be clustered together in another clone, as expected from the data of Figure 14.

Cloning of the KLK-L3 gene

A putative new gene, formed of three exons, was predicted by computer analysis of the genomic sequence. The predicted exons were subjected to sequence homology search against the human EST database (dbEST) and revealed an EST clone (GenBank accession # AA583908) which exhibited 99% homology with the putative gene. This EST was obtained, purified and sequenced and the sequence was aligned by BLAST software (37) against the genomic area that contains the putative gene. An additional exon, downstream of the predicted structure, was identified. The 3' end of the gene was verified by: (a) The presence of the serine residue (S) of the catalytic triad in a well-conserved region. This highly conserved motif (GDSGGP) always occurs at the beginning of the last exon in all known kallikreins. (b) The presence of a stop codon that is in frame with the predicted amino acid sequence. (c) The presence of a 19-poly A stretch at the end of the EST that was not found in the genomic sequence.

To verify the accuracy of the cDNA sequence of the gene, PCR reactions were performed using gene-specific primers for the first and last exons of the predicted structure of the gene (L3-F2 and L3-R1) with cDNA isolated from different human tissues as putative templates. A positive band of the expected

size was isolated from testis cDNA and fully sequenced. Its sequence was aligned by BLAST against the genomic sequence to unequivocally define the exon/intron boundaries. For further characterization of the 5' end of the gene, 5'RACE reaction was performed using Marathon Ready cDNA from testis as a template. This allowed identification of an additional exon that contains the start codon and 5' untranslated region. The full sequence of the gene is shown in SEQ. ID. NO. 21 (GenBank Accession # AF135026) and the amino acid sequences of KLK-L3 proteins are shown in SEQ. ID. Nos. 22 and 23.

Structural characterization of the KLK-L3 gene:

As shown in Figure 15, the KLK-L3 gene is formed of 5 coding exons and 4 intervening introns, although, as with other kallikreins, the presence of further upstream untranslated exon(s) could not be ruled out (14, 28). All of the exon /intron splice sites conform to the consensus sequence for eukaryotic splice sites (32). The gene further follows strictly the common structural features of the human kallikrein multigene family, as described below.

The predicted protein-coding region of the gene is formed of 753 bp, encoding a deduced amino acid polypeptide with a predicted molecular weight of 27.5 kDa. A potential translation initiation codon is found at position 28 of the predicted first exon (numbers of nucleotides refer to SEQ. ID. NO. 21 or GenBAnk Accession # AF135026. This codon does not match well with the consensus Kozak sequence (33), however, it has a purine at position (-3) which occurs in 97% of vertebrate mRNAs (43), and it is almost identical to the sequence of the zyme gene flanking the start codon. It should also be noted that most kallikreins do not have the consensus G nucleotide in position (+4).

Nucleotides 6803-6808 (AGTAAA) closely resemble a consensus polyadenylation signal (44) and are followed by a stretch of 19 poly A nucleotides not found in genomic DNA, after a space of 14 nucleotides. No other potential polyadenylation signals were discernable in the 3' untranslated region, suggesting that the above motif is indeed the polyadenylation signal. The same polyadenylation signal motif was predicted for the KLK1 and KLK2 genes.

Although the KLK-L3 protein sequence is unique, comparative analysis revealed that it is highly homologous to other members of the kallikrein multigene family. KLK-L3 shows 40% protein identity with the TLSP gene product and 38% and 33% identity with the KLK-L2 and KLK1 proteins, respectively. Hydrophobicity analysis revealed that the amino-terminal region is quite hydrophobic (Figure 16), consistent with the possibility that this region may harbor a signal sequence, analogous to other serine proteases. Computer analysis of the aminoacid sequence of KLK-L3 predicted a cleavage site between amino acids 19 and 20 (GWA-DT). Sequence alignment (Figure 17) also revealed a potential cleavage site (Arg^{22}), at a site homologous to other serine proteases (lysine (K) or arginine (R) is present in most cases). Several evenly distributed hydrophobic regions throughout the KLK-L3 polypeptide are consistent with a globular protein, similar to other kallikreins and serine proteases. The dotted region in Figure 17 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L3 or other members of the kallikrein multi-gene family (11, 41).

Twenty nine "invariant" amino acids surrounding the active site of serine proteases have been described. Of these, twenty-six are conserved in KLK-L3. One of the unconserved amino acids (Ser^{168}

instead of Pro) is also found in prostase, KLK-L2 and enamel matrix serine proteinase (EMSP1) genes. The second (Leu^{58} instead of Val) is also found in TLSP and KLK-L2 genes, and the third is Ala^{26} instead of Gly. According to protein evolution studies, each of these changed amino acids represents a conserved evolutionary change to a protein of the same group (45). Twelve cysteine residues are present in the putative mature KLK-L3 protein, ten of them are conserved in all the serine proteases that are aligned in Figure 17, and would be expected to form disulphide bridges. The other two (C^{136} and C^{238}) are not found in PSA, KLK1, KLK2 or trypsinogen; however, they are found in similar positions in prostase, HSCCE, zyme neuropsin, and TLSP genes and are expected to form an additional disulphide bond.

To predict the phylogenetic relatedness of the KLK-L3 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA method (Figure 18). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped KLK-L3 with TLSP, neuropsin, zyme, HSCCE and prostase/KLK-L1, consistent with previously published studies (11, 41).

Tissue expression and hormonal regulation of the KLK-L3 gene

As shown in Figure 19, the KLK-L3 gene is primarily expressed in thymus, testis, spinal cord, cerebellum, trachea, mammary gland, prostate, brain, salivary gland, ovary and skin (the latter two tissues are not shown in the figure). Lower levels of expression are seen in fetal brain, stomach, lung, thyroid, placenta, liver, small intestine, and bone marrow. No expression was seen in uterus, heart, fetal liver, adrenal gland, colon, spleen, skeletal muscle, pancreas, and kidney. In order to verify the RT-PCR specificity, representative PCR products were cloned and sequenced. Figure 20 shows that KLK-L3 gene is regulated by steroid hormones in the human breast cancer cell line BT-474.

DISCUSSION

A human kallikrein gene locus has been defined, and the first detailed map describing the relative positions of the kallikreins and other kallikrein-like genes has been constructed (Figure 14). This map is consistent with previous reports on the localization of the classical kallikreins and the approximate mapping of some new kallikreins by radiation hybrid and FISH techniques (9, 14, 67). It should be noted, however, that the lengths of certain segments of this map (as depicted in Figure 14) are dependent on the EcoR1 restriction map of the area and are measured in terms of approximate kb units. Also, the measure of intervals between genes may change slightly in the future, since some kallikreins may have extra 5'exon(s) that have not as yet been identified. Kallikreins with verified 5'-untranslated exons include NES1 (14), zyme, and neuropsin (35). This map is also directional; it indicates that PSA and KLK2 genes are transcribed in the same direction (centromere to telomere) and that the rest of the kallikrein-like genes are transcribed in the reverse direction (Figure 14).

An early report indicated that KLK1 is located approximately 31 kb centromeric to PSA (9). The map described extends only 24 kb centromeric to PSA, and for this reason, KLK1 was not precisely localized. Thus, the exact location of the KLK1 gene is still to be defined from linear chromosome 19 sequencing data. The possibility still exists that this locus is extended further, and that other kallikrein-like

genes may be located upstream of PSA or downstream from TLSP.

Traditionally, kallikreins are characterized by their ability to liberate lysyl-bradykinin (kallidin) from kininogen (2). In humans, only KLK1 meets this "functional" definition. KLK2 and KLK3 are assigned to the same family based on the strong structural similarities of the genes and proteins and the close localization of these genes on the same chromosomal region (20). More recently, a new structural concept has emerged to describe kallikreins. Richards and co-workers introduced the concept of a "kallikrein multigene family" in mice, to refer to these genes (20, 21). This definition is not based much on the specific enzymatic function of the gene product, but more on its sequence homology and its close linkage on mouse chromosome 7.

Irwin et al. (27) proposed that the serine protease genes can be classified into five different groups according to intron position as discussed above.The results indicate the presence of some more common structural features that are found in all kallikreins (including the newly identified KLK-L3 gene) : (1) All genes are formed of 5 coding exons and 4 intervening introns (with the possibility that some genes may have extra 5' untranslated exon(s) (24, 31, 35) (Figure 21). (2) The exon lengths are usually comparable. (3) The intron phases are always conserved (I-II-I-0) (see Figure 21 for description of intron phases). (4) These genes are clustered in the same chromosomal region, apparently without any intervening non kallikrein-like genes (Figure 14). Thus, all the recently identified serine proteases that are present in this region (zyme, HSCCE, neuropsin, NES1, prostase/KLK-L1, KLK-L2 and TLSP), together with the newly identified kallikrein-like gene (KLK-L3), could be considered members of the expanded human kallikrein multigene family.

The chromosomal band 19q13 is nonrandomly rearranged in a variety of human solid tumors including ovarian cancers (46), and the currently available data indicate that the kallikrein gene locus is related to many malignancies. At least three kallikrein genes (PSA, zyme and NES1) are down regulated in breast cancer (10, 13, 47, 48), and NES1 appears to be a novel tumor suppressor gene (29). Furthermore, PSA exhibits potent antiangiogenic activity (49). It is possible that some of these kallikreins are involved in a cascade pathway, similar to the coagulation or apoptotic process, whereby pro-forms of proteolytic enzymes are activated and then act upon downstream substrates. Such activity was found for the KLK2 gene product which acts upon and activates pro PSA (50, 51).

The expanded human kallikrein gene family has similar number of members as the rodent family of genes. Some new compelling data have raised the possibility that at least some of these genes behave as tumor suppressors (29), as negative regulators of cell growth (52), as antiangiogenic (49) and apoptotic (53) molecules. The paramount diagnostic value of some members is also well-established (24, 54). For these reasons, it is important to check all members of this family of genes as potential diagnostic or prognostic markers or as candidate therapeutic targets.

The newly identified KLK-L3 gene is expressed in many tissues, including skin, thymus, central nervous system, breast, prostate, and testis. The wide range of tissue expression of KLK-L3 should not be surprising since, by using the more sensitive RT-PCR technique, many kallikrein genes were found to be expressed in a wide variety of tissues. For example, PSA, KLK2, prostase/KLK-L1, and KLK-L2 are now

known to be expressed in breast and many other tissues (30, 54).

Like many other kallikreins, KLK-L3 is regulated by steroid hormones but in a more complex fashion than PSA and KLK2 which are up-regulated by androgens and progestins (71). In the breast carcinoma cell line studied, KLK-L3 appears to be up-regulated by progestins > estrogens > androgens (Figure 20).

Example 5

Materials and Methods

DNA sequence on chromosome 19 and prediction of new genes

Sequencing data of approximately 300Kb of nucleotides, around chromosome 19q13.3-q13.4, was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (http://www http://www-bio.llnl.gov/genome/genome.html) and an almost contiguous stretch of genomic sequences was constructed. A number of computer programs were used to predict the presence of putative new genes in this genomic area.

Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (http://www ncbi.nlm.nih.gov/BLAST/) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL. The clones were propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Rapid amplification of cDNA ends (3' RACE)

According to the EST sequence data and the predicted structure of the gene, two gene-specific primers were designed and two rounds of RACE reactions (nested PCR) were performed with 5µl Marathon Ready™ cDNA of human testis (Clontech, Palo Alto, CA, USA) as a template. The reaction mix and PCR conditions used were according to the manufacturer's recommendations.

Tissue expression

Total RNA isolated from 26 different human tissues was purchased from Clontech. cDNA was prepared as described below, and used for PCR reactions with different sets of primers (Table 15, SEQ.ID.NOs. 46-55, 9-12). Tissue cDNAs were amplified at various dilutions.

Breast cancer cell line and hormonal stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls.

The cells were cultured for 24 hours, then harvested for mRNA extraction.

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the breast cancer tissues and cell lines using Trizol™ reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 μg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 μl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (L4-F1 and L4-R1, see Table 15, SEQ.ID.NOs. 46 and 47) and PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Normal and malignant breast tissues

Normal breast tissues were obtained from women undergoing reduction mammoplasties. Breast tumor tissues were obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The normal and tumor tissues were immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extracted. The tissues were pulverized with a hammer at dry ice temperature and RNA was extracted as described above, using Trizol reagent.

Structure analysis

Multiple alignment was performed using the Clustal X software package available at: ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/ clustalx/[clustalx1.64b.msw.exe] and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA [kiwi.imgen.bcm.tmc. edu:8808/search-launcher/launcher/html]. Phylogenetic studies were performed using the Phylip software package available at: http://evolution.genetics. washington.edu/phylip/getme.html. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs [http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html]. Signal peptide was predicted using the "SignalP" server [http://www.cbs. dtu.dk/services/ signal]. Protein structure analysis was performed by the "SAPS" (structural analysis of protein sequence) program [http://dot.imgen.bcm.tmc.edu: 9331/seq-search/struc-predict.html].

Results

Cloning of the KLK-L4 gene

Computer analysis of the genomic sequence around chromosome 19 q13.3-q13.4 predicted a putative new gene formed of at least 3 exons. To experimentally verify the existence of this gene, the putative exons were subjected to sequence homology search against the human expressed sequence tag (EST) database (dbEST), and four EST clones with > 97% homology were identified (Table 16). All ESTs were cloned from testicular tissue. These clones were obtained and inserts were sequenced from both directions. Sequences were then compared with the computer-predicted structure and final selection of the intron/exon splice sites was made according to the EST sequences.

As shown in Figure 22, three ESTs match almost perfectly with the predicted 3 exons (exons 3, 4, 5) of the gene and one EST matches perfectly with predicted exons 3 and 5. However, each of the ESTs extends further upstream with different exonic patterns, suggesting the presence of different splice variants. Attempts to translate these clone sequences demonstrated the presence, in some ESTs, of interrupting stop codons in all three possible reading frames. A homology search of the three common exons against the GenBank database revealed a cDNA sequence from the German Human Genome Project. This clone has an identical exon 2 as the long form of KLK-L4 gene [this form will be described below] but has an extended exon 3 that ends with a stop codon (Figure 22). This clone was isolated from uterine tissue and is translated by software into a truncated protein product of 196 amino acids which is followed by a 3' untranslated region [GenBank accession # AL050220].

Screening of cDNAs from 26 different tissues by RT-PCR, using gene-specific primers for exons 3 and 5 [L4-F1 and L4-R1] (Table 15 & Figure 22) revealed that this gene is expressed in many tissues. Four tissues that show the highest level of expression [salivary gland, mammary gland, prostate, and testis] (Figure 23) and uterus [the EST clone AL050220 was isolated from this tissue] were selected for identification of the full structure of the gene. Different PCR reactions were performed using one reverse primer (L4-R1) together with each of the forward primers located in upstream exons that were found in the different EST clones [primers L4-B, L4-D, L4-E] (Table 15 & Figure 22). The PCR reactions were performed under different experimental conditions, using the EST clones as positive controls, and the PCR products were sequenced. None of these forms were found in any of the tissues, except in testis where all three forms were found.

By RT-PCR of the KLK-L4 gene using primers L4-R1 and L4-F1, it was found that the gene is expressed in a wide variety of tissues (Figure 23). In order to obtain the structural forms that exist in these tissues, a homology study was performed. Aligning the predicted polypeptide of the KLK-L4 gene with all other kallikreins and kallikrein-like genes, suggested, by homology, that at least two more exons should be present upstream of the predicted three exons. The genomic fragment upstream of the third exon was subjected to further computer analysis for gene prediction, and exon 2 was identified based on: a) a consensus exon/intron splice site b) preservation intron phase II after this exon, in agreement to intron phases of all other known kallikreins c) presence of the histidine residue of the catalytic triad (H^{76}) surrounded by a well-conserved peptide motif [see below] just before the end of this exon d) comparable exon length to other kallikrein genes. A potential first exon was also predicted from the upstream genomic

sequence, based on the preserved intron phase (phase I), and the existence of an in-frame start codon that is located at a comparable distance [in relation to other kallikreins] from the end of this exon. To verify this predicted structure, a PCR reaction was performed using one reverse primer (L4-R1) together with another forward primer that is located in the predicted first exon (primer L4-X1) (Table 15 & Figure 22). Two main PCR bands were obtained from the tissues examined; the expected 819 bp band (predominant) and an additional minor band of about 650 bp (Figure 24). Cloning and sequencing of these two bands revealed that the gene exists in two main forms in these tissues; the long form [SEQ. ID. No. 43 or GenBank Accession No. AF135024] and another form [referred to as the short KLK-L4 variant] that utilizes an upstream alternative splice donor site, located inside exon 3, thus creating an mRNA product that that is 214 bp shorter. This alternative splice site causes frame-shifting of the coding region that will generate a predicted stop codon at the beginning of exon 4, giving rise to a truncated protein product that does not contain the serine residue of the catalytic triad (Figures 24 and 25).

Aligning the long KLK-L4 form with the ESTs (Figure 22) demonstrated that all ESTs utilize a different splice donor site located 80 bp downstream from the end of exon 3. These additional 80 bp contain an in-frame stop codon at nucleotide position 5505 which will lead to the formation of a shorter polypeptide product. They also utilize an alternative polyadenylation signal located at position 8706 [numbers refer to SEQ. ID. No. 43 or or GenBank Accession No. AF135024]. The clone from the German Genome Project utilizes another splice donor site that is located further downstream, inside intron 3, and ends up with a poly A tail without having a fourth or fifth exon. The same stop codon (position 5505) will be in-frame, and therefore, a truncated protein product is predicted to be formed (Figure 22).

In order to obtain the 3'end of the gene, a 3'RACE reaction was performed, and an additional 375 bp fragment of 3' untranslated region, downstream from PCR primer L4-R1, was obtained. This fragment was further confirmed to be present in all tissues tested, by performing a PCR reaction using primers L4-F1 and L4-R3 (Table 15 & Figure 22). This fragment ends with a putative polyadenylation signal variant (TATAAA).

Structural characterization of the KLK-L4 gene and its protein product

The long form of the KLK-L4 gene is presented in Figure 25 (SEQ.ID.NO. 43). KLK-L4 is formed of five coding exons and four intervening introns, spanning an area of 8,905 bp of genomic sequence on chromosome 19q13.3-q13.4. The lengths of the coding regions are 52, 187, 269,137 and 189 bp, respectively. The predicted protein coding region of the gene is formed of 831 bp, encoding a deduced 277-amino acid protein with a predicted molecular mass of 30.6 kDa (Figure 25). The intron/exon splice sites (mGT....AGm, where m is any base) and their flanking sequences are in agreement with the consensus splice site sequence. A potential translation initiation codon is present at position 45 of the predicted first exon [numbers refer to SEQ. ID. No. 43]. The cDNA extends at least 382 bp further downstream from the stop codon and a putative polyadenylation signal (TATAAA) is present at the end of this region (Figure 25).

Hydrophobicity analysis revealed that the amino-terminal region is quite hydrophobic (Figure 26), consistent with the possibility that this region may harbor a signal sequence, analogous to other serine

proteases. Figure 26 also shows the presence of several evenly distributed hydrophobic regions throughout the KLK-L4 polypeptide, which are consistent with a globular protein, similar to other serine proteases (13). Computer analysis of the amino acid sequence of KLK-L4 predicted a cleavage site between amino acids 20 and 21 (GVS-QE). Sequence homology with other serine proteases (Figure 27) predicted another potential cleavage site (Lys25) in close proximity. Most other kallikreins are activated by cleavage after arginine or lysine. Thus, the protein product is very likely to be a secreted protein. The dotted region in Figure 27 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) which is not found in KLK-L4 or other members of the kallikrein multi-gene family (11,13, 35).

Amino acid sequences for KLK-L4 proteins are shown in SEQ.ID.NO. 44 and 45.

Sequence analysis of eukaryotic serine proteases indicates the presence of twenty nine invariant amino acids (39). Twenty eight of them are conserved in the KLK-L4 protein and the remaining amino acid (Q182 instead of P) is not conserved among all other kallikreins (Figure 27). Ten cysteine residues are present in the putative mature KLK-L4 protein. These are conserved in all the serine proteases that are aligned in Figure 27, and would be expected to form disulphide bridges. The presence of aspartate (D) in position 239 suggests that KLK-L4 will possess a trypsin-like cleavage pattern, similarly to most of the other kallikreins [e.g., KLK1, KLK2, TLSP, neuropsin, zyme, prostase, and EMSP] but different from PSA which has a serine (S) residue in the corresponding position, and is known to have chymotrypsin like activity (Figure 27) (2,40).

Mapping and chromosomal localization of the KLK-L4 gene

Alignment of the KLK-L4 gene and the sequences of other known kallikrein genes within the 300 Kb area of interest [the human kallikrein gene family locus], enabled precise localization of all known genes and to determine the direction of transcription, as shown by the arrows in Figure 28. The PSA gene lies between KLK1 and KLK2 genes and is separated by 13, 319 base pairs (bp) from KLK2, and both genes are transcribed in the same direction (centromere to telomere). All other kallikrein-like genes are transcribed in the opposite direction. KLK-L4 is 13 kb centromeric from KLK-L6 [SEQ.ID.NO. 65], and 21 kb more telomeric to KLK-L5 [SEQ. ID. NO. 56].

Homology with the kallikrein multi-gene family

Alignment of the amino acid sequence of the KLK-L4 protein (long form) against the GenBank database and the known kallikreins, using the BLAST algorithm (37), indicated that KLK-L4 has 51% amino acid sequence identity with the TLSP and zyme genes, 49% identity with KLK-L2 and 47% and 45% identity with PSA and KLK2 genes, respectively. Multiple alignment study shows that the typical catalytic triad of serine proteases is conserved in the KLK-L4 gene (H^{108}, D^{153}, and S^{245}) and, as is the case with all other kallikreins, a well conserved peptide motif is found around the amino acid residues of the catalytic triad [i.e. histidine (WLLTAAHC), serine (GDSGGP), and aspartate (DLMLI)] (Figure 27) (1, 11, 13, 35). In addition, several other residues were found to be fully or partially conserved among the human kallikrein gene family, as further shown in Figure 27. To predict the phylogenetic relatedness of the KLK-L4 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-

joining/UPGMA method (Figure 29). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped KLK-L4 with zyme, TLSP, KLK-L3, neuropsin, and NES1 genes, consistent with previously published studies (41) and indicating that this group of genes probably arose from a common ancestral gene by duplication.

Tissue expression and hormonal regulation of the KLK-L4 gene

As shown in Figure 23, the KLK-L4 gene is primarily expressed in mammary gland, prostate, salivary gland and testis, but, as is the case with other kallikreins, lower levels of expression are found in many other tissues. In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced.

A steroid hormone receptor-positive breast cancer cell line (BT-474) was used as a model, to verify whether the KLK-L4 gene is under steroid hormone regulation. PSA was used as a control gene, known to be up-regulated by androgens and progestins and pS2 as an estrogen up-regulated control gene in the same cell line. Preliminary results indicate that KLK-L4 is up-regulated by progestins and androgens and to a lower extent by estrogens (Figure 30).

Expression of KLK-L4 in breast cancer tissues and cell lines

To characterize the extent and frequency of expression of the KLK-L4 gene in breast tumors, cDNA derived from 3 normal and 19 malignant breast tissues and 3 breast cancer cell lines was used. The data were interpreted by comparison of band intensities. Out of the 19 tumors, KLK-L4 gene expression was undetectable in 7, lower than normal tissues in 9, comparable to the normal tissues in 1, and higher than normal tissues in 2 tumors. Without hormonal stimulation, the BT-474 and T-47D cell lines had no detectable KLK-L4 mRNA, while the MCF-7 cell line was positive. These preliminary results suggest that this gene is down-regulated in the majority (16/19) of breast tumors.

Discussion

The established kallikreins (KLK1, KLK2, and PSA), trypsinogen and chymotrypsinogen belong to a group that has: (1) an intron just downstream from the codon for the active site histidine residue, (2) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (3) a third intron just upstream from the exon containing the codon for the active site serine residue. Figure 31 shows that KLK-L4 meets the above mentioned criteria; moreover, is located in close proximity to other kallikrein genes on the chromosomal locus 19q13.3-q13.4 (Figure 28).

The preliminary findings, supporting that the KLK-L4 gene may be down-regulated in a subset of breast cancers, is not surprising. There is now growing evidence that many of the kallikreins and kallikrein-like genes that are clustered in the same chromosomal region (Figure 28) are related to malignancy. PSA is the best marker for prostate cancer so far (24). A recent report provided evidence that PSA has antiangiogenic activity, and that this activity may be related to its function as a serine protease (49). This study suggested that other serine proteases, including new members of the kallikrein multigene family of enzymes, should also be evaluated for potential antiangiogenic activity (49). Recent reports suggest that hK2 (encoded by the KLK2 gene) could be another useful diagnostic marker for prostate cancer (57, 58). NES1 appears to be a tumor suppressor gene (29). The protease M gene was shown to

be differentially expressed in primary breast and ovarian tumors (10), and the human stratum corneum chymotryptic enzyme has been shown to be expressed at abnormally high levels in ovarian cancer (59). Another recently identified kallikrein-like gene, located close to KLK-L4 and tentatively named tumor-associated, differentially expressed gene-14 (TADG14) [an alternatively spliced form of neuropsin, see Figure 28] was found to be overexpressed in about 60% of ovarian cancer tissues (59). Also, prostase/KLK-L1, another newly discovered kallikrein-like gene, is speculated to be linked to prostate cancer (41). Thus, extensive new literature suggests multiple connections of many kallikrein genes to various forms of human cancer.

The removal of intervening RNA sequences (introns) from the pre-messenger RNA in eukaryotic nuclei is a major step in the regulation of gene expression (60). RNA splicing provides a mechanism whereby protein isoform diversity can be generated and the expression of particular proteins with specialized functions can be restricted to certain cell or tissue types during development (60). The sequence elements in the pre-mRNA at the 5' and 3' splice sites in metazoans have very loose consensus sequence; only the first and the last two bases (GT..AG) of the introns are highly conserved (Sambrook, supra). These sequences cannot be the sole determinants of splice site selection, since identical, but not ordinarily active, consensus sequences can be found within both exons and introns of many eukaryotic genes. Other protein factors and sequences downstream of the splice sites are also involved.

The existence of multiple splice forms is frequent among kallikreins. Distinct RNA species are transcribed from the PSA gene, in addition to the major 1.6-kb transcript (61). Several distinct PSA transcripts have been described by Reigman et al (7). Interestingly, one of these clones lacks the 3' untranslated region and the first 373 nucleotides of the open reading frame, and has an extended exon that contains a stop codon, a pattern that is comparable with some alternative forms of the KLK-L4 cDNA, as described here (Figure 22). Heuze et al., reported the cloning of a full-length cDNA corresponding to a 2.1 kb PSA mRNA. This form results from the alternative splicing of intron 4 and lacks the serine residue that is essential for catalytic activity (61). Also, Reigman et al reported the identification of two alternatively spliced forms of the human glandular kallikrein 2 (KLK2) gene (62). A novel transcript of the tissue kallikrein gene (KLK1) was also isolated from the colon (63). Interestingly, this transcript lacks the first two exons of the tissue kallikrein gene, but the last three exons were fully conserved, a pattern that is similar to the findings with some ESTs containing parts of the KLK-L4 gene (Figure 22). Neuropsin, a recently identified kallikrein-like gene, was found to have two alternatively spliced forms, in addition to the major form (59, 64). Here, the cloning of the KLK-L4 gene is described and the identification of a number of alternative mRNA forms. These forms may result from alternative splicing (Sambrook, supra), retained intronic segment (7), or from the utilization of an alternative transcription initiation site (63). Because the long form of KLK-L4 and the major alternative splice variant [short KLK-L4 variant] (Figure 24) have an identical 5' sequence required for translation, secretion and activation, it is possible to assume that both mRNAs encode for a secreted protein (61).

In order to investigate the relative predominance of the long KLK-L4 and related forms, cDNA from various tissues was amplified by PCR. Although, in general, it is difficult to use PCR for quantitative

comparisons between mRNA species, in this experiment, [mRNAs of comparable sizes, using one set of primers under identical conditions], such a comparison is reasonable (62). In all five normal tissues examined [breast, prostate, testis, salivary gland and uterus] the long form of KLK-L4 was the predominant, with minimal level of expression of the short form (Figure 24).

The presence of alternatively spliced forms may be related to malignancy. Recent literature suggests that distinct molecular forms of PSA could be expressed differently by malignant versus benign prostate epithelium (65). Aberrant PSA mRNA splicing in benign prostatic hyperplasia, as opposed to prostate cancer, has been described by Henttu et al (66). In addition, it has been recently postulated that different prostatic tissues potentially harboring occult cancer could account for the presence of various forms of PSA (65).

Example 6

Materials and Methods

DNA sequence on chromosome 19

Sequencing data of approximately 300Kb of nucleotides on chromosome 19q13.3-q13.4 was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (http://www-bio.llnl.gov/genome/genome.html). This sequence was in the form of 9 contigs of different lengths. Restriction enzyme analysis, long PCR strategies, followed by DNA sequencing, BAC and PAC library screening and end sequencing of selected clones, were used to construct a contiguous genomic region, representing the complete human kallikrein gene locus.

New gene identification

A number of computer programs were used to predict the presence of putative new genes within the contiguous genomic area of interest. The ability of these programs for predicting new genes was first examined by using the genomic sequences of the known kallikreins as testing parameters. The most reliable computer programs; GeneBuilder (gene prediction) (http://125.itba.mi.cnr.it/ ~webgene/genebuilder.html), GeneBuilder (exon prediction) (http://125.itba.mi.cnr.it/~webgene/genebuilder.html), Grail 2 (http://compbio.ornl.gov), and GENEID-3 (http://apolo.imim.es/geneid.html) were selected for further use.

Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (http://www ncbi.nlm.nih.gov/BLAST/) against the human EST database (dbEST). A clone with > 95% homology was obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL. This clone was propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Rapid amplification of cDNA ends (RACE)

According to the EST sequence and the predicted structure of the gene, two sets of gene-specific primers were designed for 5' and 3' RACE reactions. Two rounds of RACE reactions (nested PCR) were performed for each type of RACE with 5µl Marathon Ready™ cDNA of human testis and prostate (Clontech, Palo Alto, CA, USA) as templates. The reaction mix and PCR conditions were selected

according to the manufacturer's recommendations. In brief, the initial denaturation was for 5 min at 94°C, followed by 94°C for 5 s and 72°C for 2 min, for 5 cycles; then, 94°C for 5 s and 70° C for 2 min, for 5 cycles; then, 94°C for 5 s and 65°C for 2 min for 30 cycles for the first reaction and 25 cycles for the nested PCR reaction.

Tissue expression

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described below for the tissue culture experiments and used for PCR reactions. After aligning all known kallikrein genes, two primers (KLK-L5-R1 and KLK-L5-F1) (Table 17, SEQ.ID.NOs. 61-64, 9-12, and Figure 32) were designed from areas with relatively low homology. Tissue cDNAs were amplified at various dilutions. Due to the high degree of homology between kallikreins, and to exclude non-specific amplification, PCR products were cloned and sequenced.

Normal and malignant breast tissues

Normal breast tissues were obtained from women undergoing reduction mammoplasties. Breast tumor tissues were obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The normal and tumor tissues were immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extracted. The tissues were pulverized with a hammer under liquid nitrogen and RNA was extracted as described below, using Trizol reagent.

Breast and prostate cancer cell lines and hormonal stimulation experiments

The breast cancer cell lines BT-474 and T-47D, and the LNCaP prostate cancer cell line were purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cell lines or tissues using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 μg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 μl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (KLK-L5-F1 and KLK-L5-R1) (Table 17) and PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling

conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension step at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Structure analysis

Multiple alignment was performed using the Clustal X software package available at: ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/ (clustalx1.64b.msw.exe) and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA (kiwi.imgen.bcm.tmc. edu:8808/search-launcher/launcher/html). Phylogenetic studies were performed using the Phylip software package available at: http://evolution.genetics. washington.edu/phylip/getme.html. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html). Signal peptide was predicted using the "SignalP" server (http://www.cbs.dtu.dk/services/ signal). Protein structure analysis was performed by "SAPS" (structural analysis of protein sequence) program (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html).

RESULTS

Identification of the KLK-L5 gene

Computer analysis of the genomic area of interest (300Kb around chromosome 19q13.3-q13.4) predicted a putative gene comprised of at least three exons. Screening of the human expressed sequence tag (EST) database revealed an EST clone (GenBank Accession #394679) with 99% homology with the predicted exons. This clone was obtained, purified, and sequenced. The full-length sequence of the EST was compared with the genomic area containing the putative new gene and showed 100% homology with certain areas (exons), which were separated by introns. This alignment indicated that the new gene was comprised of 7 exons. Sequence homology comparisons and phylogenetic analysis revealed that this new gene is structurally similar to known kallikreins and other serine proteases (see below). Since four other new kallikrein-like genes were discovered in this area, this gene was named KLK-L5 (for kallikrein-like gene 5). Attempts to translate the coding region in all three possible reading frames indicated that only one reading frame will produce a full-length polypeptide chain without interrupting in-frame stop codons. Further support for the correctness of this reading frame was obtained by demonstrating that only this frame will preserve the three amino acid residues necessary for serine protease activity (catalytic triad) and the conserved motifs around them. An in-frame methionine start codon was found in the second exon. This start codon falls within a typical consensus Kozak sequence (CCACC**ATG**G) (33). Thus, the gene will have at least one 5' untranslated exon, similarly to other kallikrein-like genes [e.g. zyme, the normal epithelial cell-specific 1 gene (NES1) (14), and neuropsin (35)]. 5' and 3' RACE reactions were performed in order to

obtain the 5' and 3' ends of the gene. No more sequence was obtained by 5' RACE. However, 3' RACE enabled identification of the 3' untranslated region of the gene. The additional sequence ends with a poly-A stretch that does not exist in the genomic structure, thus marking the 3' end of the gene and the start of the poly-A tail.

Splice variants of the KLK-L5 gene

Homology analysis of the KLK-L5 gene with other kallikreins revealed the presence of an additional 3' exon, an observation that has not been reported for any other member of the kallikrein multigene family. Furthermore, two different PCR bands were obtained with the 3' RACE. Sequencing of these bands revealed that this gene has at least two splice forms at its 3' end; one form in which the last exon is a single continuous fragment, and another form in which the last exon is split into two exons, with an intervening intron. In order to identify the full structure of other possible splice variants of the gene, PCR was performed using two primers (L5-F2 and L5-R2) (Table 17 and Figure 32). cDNA from 26 different tissues were used as templates and the reaction was performed under different experimental conditions (annealing temperature, $MgCl_2$ concentration). Three distinct bands were observed in many tissues. These bands were excised, gel-purified, and sequenced. As shown in Figure 32, the KLK-L5 gene was found to have 3 molecular forms:

1) One form (referred to, from now on, as the "classical" form) represents a typical kallikrein-like serine protease with five coding exons and four intervening introns (Figure 32). As is the case with some other kallikreins, a 5' untranslated exon is also present, and the possibility of further upstream untranslated exon(s) could not be excluded. Exons 1, 2 and 3 were present at the aforementioned EST. The start codon is present in the second exon (numbers refer to SEQ.ID.NO. 56 or GenBank Accession # AF135025). The stop codon is located in the sixth exon, followed by a 3' untranslated region, and a typical polyadenylation signal (AATAAA) is located 16 bp before the poly-A tail (Figure 33). This form of KLK-L5 spans a genomic length of 5,801 bp on chromosome 19q13.3-q13.4. The lengths of the coding regions of the exons are 37, 160, 260, 134, and 156 bp, respectively (Figures 33 and 34). The predicted protein-coding region is formed of 747 bp, encoding a deduced 248-amino acid protein with a predicted molecular mass of 26.7 kDa. The intron/exon splice sites (GT....AG) and their flanking sequences are in agreement with the consensus splice site sequence.

2) The second mRNA form, encoding the KLK-L5-related protein-1, is an alternatively spliced form in which the last exon is split into two separate exons with an additional intervening intron (Figure 32). This splitting of the last exon results in the utilization of another stop codon at position 9,478, thus creating a deduced 254-amino acid protein that is 6 amino acids longer than the "classical" KLK-L5 form and its carboxyterminal end is different in sequence by 19 amino acids (Figure 32). This variant has a predicted molecular mass of 27.1 kDa (for base numbering please see SEQ.ID.NO. 56 and GenBank Accession # AF135025).

3) The third mRNA form, encoding for KLK-L5-related protein-2, is similar to the classical form except that the fourth exon is missing (Figure 32). This leads to frame shifting of the coding region, and an earlier in-frame stop codon will be encountered at position 9,180. The protein-coding region of this form

consists of 336 bp, encoding for a predicted 111-amino acid protein with a molecular mass of 12 kDa. This protein will lack both the serine and aspartate residues characteristic of serine proteases.

Amino acid sequences for KLK-L5 proteins are shown in SEQ. ID. NOs. 57 to 60.

Structural analysis of the classical KLK-L5 gene

Figure 35 shows a comparative hydrophobicity analysis of the KLK-L5 and the prostate-specific antigen (PSA) proteins. The amino terminal regions of both genes are quite hydrophobic, indicating that this region of KLK-L5 is possibly harboring a signal peptide analogous to PSA. Figure 35 also shows the presence of several evenly distributed hydrophobic regions throughout the KLK-L5 polypeptide, which are consistent with a globular protein, similar to other serine proteases (13). Figure 36 shows the alignment of KLK-L5 protein with another 10 members of the same gene family. The dotted region in Figure 36 indicates an 11-amino acid loop characteristic of the classical human kallikreins (PSA, hK1 and hK2) but not found in KLK-L5 protein or other members of the kallikrein multigene family (11, 13, 35). Sequence analysis of eukaryotic serine proteases indicates the presence of twenty nine invariant amino acids (39). Twenty eight of them are conserved in the KLK-L5 polypeptide and the remaining amino acid (S156 instead of P) is not conserved among all other kallikreins (Figure 36). Twelve cysteine residues are present in the putative mature KLK-L5 protein, ten of them are conserved in all kallikreins, and the remaining two (C133, and C235) are present in most of the other kallikrein-like proteins but not in the classical kallikreins and they are expected to form an additional disulphide bridge (Figure 36).

The presence of aspartate (D) at position 194 suggests that KLK-L5 will possess a trypsin-like cleavage pattern, similarly to most of the other kallikreins (e.g., hK1, hK2, TLSP, neuropsin, zyme, prostase, and EMSP) but different from PSA, which has a serine (S) residue in the corresponding position, and is known to have chymotrypsin like activity (Figure 36) (54).

Homology with other members of the kallikrein multigene family

Although the protein encoded by the KLK-L5 gene is unique, it has a high degree of homology with the other kallikrein-like genes. The KLK-L5 protein (classical form) has 48% amino acid sequence identity and 57% overall similarity with neuropsin, 46% identity with the normal epithelial cell-specific 1 gene product (NES1) and 38% identity with both PSA and hK2 proteins. Multiple alignment shows that the typical catalytic triad of serine proteases is conserved in the KLK-L5 protein (H^{62}, D^{108}, and S^{200}) (Figures 33 and 36). In addition, a well-conserved peptide motif is found around the amino acid residues of the catalytic triad as is the case with other serine proteases [i.e., histidine (VLTAA*H*C), serine (GD*S*GGP), and aspartate (*D*LRLL)] (11, 12) (Figure 36). Figure 36 also shows other amino acid residues that are completely conserved between kallikreins and kallikrein-like proteins. To predict the phylogenetic relatedness of the KLK-L5 protein with other serine proteases, the amino acid sequences of the kallikrein proteins were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA and Protpars parsimony methods. Figure 37 shows the phylogenetic analysis which separated the classical kallikreins (hK1, hK2, and PSA) and clustered KLK-L5 with NES1 and neuropsin proteins in a separate group away from other serine proteases, consistent with previously published studies (27, 41) and indicating that this group of genes probably arose from a common

ancestral gene, by gene duplication.

Tissue expression and hormonal regulation of the KLK-L5 gene

As shown in Figure 38, the KLK-L5 gene is primarily expressed in the salivary gland, stomach, uterus, trachea, prostate, thymus, lung, colon, brain, breast and thyroid gland, but, as is the case with other kallikreins, lower levels of expression are found in some other tissues (testis, pancreas, small intestine, spinal cord). In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced. The three splice forms of the gene were expressed in most of these tissues. However, the relative abundance of each form was different among tissues (Figure 38).

In order to investigate whether the KLK-L5 gene is under steroid hormone regulation, two breast cancer cell lines (BT-474 and T-47D) and a prostate cancer cell line (LNCaP) were used as models. In LNCaP cells, the gene was up-regulated only by androgen and progestin. Only in this cell line all 3 isoforms were expressed. In BT-474 cells, KLK-L5 was found to be up-regulated, at the mRNA level, by estrogen and androgen, and to a lesser extent by the progestin. The rank of potency was estrogen>androgen>progestin. However, the rank of potency for the T-47D cell line was androgen>progestin>estrogen . In both of these cell lines, only the short isoform (related protein-2) was present (Figure 39).

KLK-L5 is down regulated in breast cancer

Expression of the KLK-L5 gene, at the mRNA level, was compared between seventeen breast cancer tissues and one normal breast tissue, by RT-PCR. The gene is not expressed at all in 12 tumors (Figure 40). In all breast tissues (normal and malignant) the short isoform (related protein-2) was predominant, with the exception of one tumor, which expressed only the classical form (Figure 40, lane 8). These results should be interpreted as preliminary, since the number of tumors and normal tissues tested is relatively small.

Mapping and chromosomal localization of the KLK-L5 gene

The knowledge of extensive genomic sequence on chromosome 19q13.3-q13.4 enabled the precise localization of 14 kallikrein-like genes and determination of the direction of transcription, as shown by the arrows in Figure 28. Only PSA and KLK2 transcribe from centromere to telomere; the rest of the genes are transcribed in the reverse direction. The KLK1 gene was found to be the most centromeric, and the KLK-L6 gene the most telomeric (KLK-L6; SEQ.ID. NO.65). KLK-L5 is 21.3 Kb centromeric to KLK-L4 (SEQ.ID.NO. 43) and 1.6 kb more telomeric to the trypsin-like serine protease gene (TLSP) (Figure 28).

DISCUSSION

As shown in Figure 34, kallikreins are characterized by the following common structural features: (a) All genes are formed of 5 coding exons and 4 intervening introns [some genes may have extra 5(untranslated exon(s)] (14, 35). (b) The exon lengths are usually comparable. (c) The intron phases are always conserved (I-II-I-0) (see legend of Figure 34 for definition of intron phases). (d) These genes are clustered in the same chromosomal region, without any intervening non-kallikrein-like genes (Figure 28). (e) The histidine residue of the catalytic triad of serine proteases is located near the end of the second coding exon; the aspartate residue in the middle of the third coding exon; and serine at the beginning of the

fifth coding exon. As shown in Figure 34, all these criteria apply to the newly identified KLK-L5 gene. Thus, KLK-L5 should be considered a new member of the kallikrein multigene family.

Serine proteases and kallikreins are synthesized as "preproenzymes" that contain an N-terminal signal peptide (pre-zymogen), followed by a short activation peptide and the enzymatic domain (41, 54). PreproPSA has 24 additional residues that constitute the pre-region (signal peptide, 17 residues), and the propeptide (7 residues) (67). The signal peptide directs the protein to and across the endoplasmic reticulum (ER). In the ER, the signal peptide is removed and the resulting proPSA is transported to the plasma membrane, where it is secreted. The hydrophobicity study (Figure 35) indicates that the amino terminal region of the KLK-L5 protein is harboring a signal peptide. Also, computer analysis of the amino acid sequence of KLK-L5 predicted a cleavage site between amino acids 17 and 18 (SQA-AT). Thus, the protein product is very likely a secreted protein.

The presence of alternatively spliced forms is not a unique feature of the KLK-L5 gene; several other kallikreins are known to be expressed in various alternatively spliced forms. In addition to the major 1.6-kb transcript, several RNA species are transcribed from the PSA gene (61). Furthermore, others (69, 70) have described several PSA isoforms. Retained introns and loss of complete exons have been reported in some of these forms. In addition, Riegman et al. reported the identification of two alternatively spliced forms of the human glandular kallikrein (KLK2) gene (62) and Liu et al. isolated three alternative forms of the same gene (68). A novel transcript of the tissue kallikrein gene was isolated from the colon (63). Neuropsin, a recently identified kallkrein-like gene, was found to have two alternatively spliced forms in addition to the major form (35, 64). Here, the cloning of the classical kallikrein form and two unique splice forms of the KLK-L5 gene are described. Because the classical form and the splice forms all have the same 5' sequence required for translation, secretion and activation as do other kallikreins, i.e. a 5' leader sequence, a signal peptide, and a proregion, it is reasonable to assume that all three mRNA forms should produce a secreted protein. Preliminary findings identifying forms of KLK-L5 predominant in certain tissues are presented in Figures 35 and 40.

The preliminary results indicate that KLK-L5 is up-regulated by steroid hormones in breast and prostate cancer cell lines (Figure 39). These results are not surprising, since many other kallikrein genes are also regulated by steroid hormones. The differences in the rank of potency of steroid hormones among different cell lines could be attributed to differences in the abundance of the steroid hormone receptors between them as described elsewhere.

In conclusion, a new member of the human kallikrein gene family, KLK-L5, has been characterized which maps to the human kallikrein locus (chromosome 19q13.3-q13.4). This gene has two related splice forms in addition to the main form. KLK-L5 is expressed in a variety of tissues, appears to be down-regulated in breast cancer and its expression is influenced by steroid hormones. Since a few other kallikreins are already used as valuable tumour markers, KLK-L5 may also find a similar clinical application.

Example 7

Using the Materials and Methods substantially as set out in Example 6, the present inventors

identified another novel gene of the kallikrein multigen family referred to as KLK-L6. The full structure of the KLK-L6 gene is shown in Figure 41. Exons 1, 2, 3, 4, 5, 6, and 7 are at nucleic acids 1172-1281; 2561-2695; 2781-2842, 3714-3885; 5715-5968; 6466-6602; and 7258-7520. The nucleic acid sequence of the KLK-L6 gene is also shown in SEQ.ID.NO. 65 and amino acid sequences for the KLK-L6 protein are shown in SEQ.ID. Nos. 66 and 67. (See also GenBank Accession # AF161221).

Figure 42 shows a comparative hydrophobicity analysis of KLK-L6 and the prostate-specific antigen (PSA). The amino terminal regions of both genes are quite hydrophobic indicating that this region of KLK-L6 is possibly harboring a signal peptide analogous to PSA

Multiple alignment of KLK-L6 was carried out using the Clustal X software program as described herein (Figure 43).

To predict the phylogenetic relatedness of the KLK-L6 protein with other serine proteases, the amino acid sequences of the kallikrein proteins were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA and Protpars parsimony methods. Figure 44 shows the phylogenetic analysis which separated the classical kallikreins (hK1, hK2, and PSA) and placed KLK-L6 in a separate group.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. All modifications coming within the scope of the following claims are claimed.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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TABLE 1. Predicted exons of the putative gene KLK-L1. The translated protein sequences of each exon (open reading frame) are shown.

Exon No.[1]	Putative coding region[2] From(bp)	To (bp)	No. of bases	Translated protein sequence	EST match[3]	Intron phase[4]	Stop codon[5]	Catalytic triad[6]	Exon prediction program[7]
2	2263	2425	163	SLVSGSCSQIINGEDCSPHSQPWQAALVMENELFCSGV LVHPQWVLSAA**H**CFQ	+	II	-	H	A,B,D
3	2847	3109	263	NSYTIGLGLHSLEADQEPGSQMVEASLSVRHPEYNRPL LAN**D**LMLIKLDESVSESDTIRSISIASQCPTAGNSCLVSG WGLLANGELT	+	I	-	D	A,B,C,D
4	3180	3317	137	GRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGG GQDQKDSCN	+	0	-		A,B,C,D
5	4588	4737	150	GD**S**GGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLC KFTEWIEKTVQAS	+	-	+	S	A,B,C

1. Conventional numbering of exons in comparison to the five coding exons of PSA,as described in Ref.14.
2. Nucleotide numbers refer to the related contig
3. (+) = >95% homology with published human EST sequences.
4.Intron phase:0=the intron occurs between codons;I=the intron occurs after the first nucleotide of the codon; II=the intron occurs after the second nucleotide of the codon.
5.(+) denotes the exon containing the stop codon.
6.H=histidine,D=aspartic acid,S=serine.The aminoacids of the catalytic triad are bold and underlined.
7. A = GeneBuilder (gene analysis), B = GeneBuilder (exon analysis), C = Grail 2, D = GENEID-3

TABLE 2. Predicted exons of the putative gene KLK-L2. The translated protein sequences of each exon (open reading frame) are shown*

Exon No.[1]	Putative coding sequence[2] From(bp)	To(bp)	No. of bases	Translated protein sequence	EST match[3]	Intron phase[4]	Stop codon[5]	Catalytic triad[6]	Exon prediction program[7]
1	15,361	15,433	73	MATARPPWMWVLCALITALLLGVT	+	I	-	-	-
2	17,904	18,165	262	EHVLANNDVSCDHPSNTVPSGSNQDLGAGAGEDARSDDSSSRIINGSDCDMHTQPWQAALLLRPNQLYCGAVLVHPQWLLTAAHCRKK	+	II	-	H	A,B,C,D
3	18,903	19,159	257	VFRVRLGHYSLSPVYESGQQMFQGVKSIPHPGYSHPGHSNDLMLIKLNRRIRPTKDVRPINVSSHCPSAGTKCLVSGWGTTKSPQ	+	I	-	D	C,D
4	19,245	19,378	134	VHFPKVLQCLNISVLSQKRCEDAYPRQIDDTMFCAGDKAGRDSCQ	+	0	-	-	B,C
5	24,232	24,384	153	GDSGGPVVCNGSLQGLVSWGDYPCARPNRPGVYTNLCKFTKWIQETIQANS	+	-	+	S	A,B,C

* All footnotes same as Table 1

TABLE 3. Predicted exons of the putative gene KLK-L3. The translated protein sequences of each exon (open reading frame) are shown*

Exon No.[1]	Putative coding region[2] From(bp)	To(bp)	No. of bases	Translated protein sequence	EST match[3]	Intron phase[4]	Stop codon[5]	Catalytic triad[6]	Exon prediction program[7]
1	70,473	70,584	112	VHFPTPINHRGGPMEEEGDGMAYHKEALDAGCTFQDP	-	I	-	-	A,B,C,D
2	70,764	70,962	199	ACSSLTPLSLIPTPGHGWADTRAIGAEECRPNSQPWQAGLF HLTRLFCGATLISDRWLLTAAHCRK	+	II	-	H	A,B,C,D
3	73,395	73,687	293	PLTSEACPSRYLWVRLGEHHLWKWEGPEQLFRVTDFFPHP GFNKDLSANDHNDDIMLIRLPRQARLSPAVQPLNLSQTCV SPGMQCLISGWGAVSSPK	+	I	-	D	A,B,C,D
4	76,305	76,441	137	ALFPVTLQCANISILENKLCHWAYPGHISDSMLCAGLWEG GRGSCQ	+	0	-	-	A,B,C,D
5	76,884	77633	749	GDSGGPLVCNGTLAGVVSGGAEPCSRPRRPAVYTSVCHYL DWIQEIMEN	-	-	+	S	A,B

* All footnotes same as Table 1

TABLE 4. Predicted exons of the putative gene KLK-L4. The translated protein sequences of each exon (open reading frame) are shown*

Exon No.[1]	Putative coding region[2] From(bp)	To(bp)	No. of bases	Translated protein sequence	EST match[3]	Intron phase[4]	Stop codon[5]	Catalytic triad[6]	Exon prediction program[7]
2	24,945	25,120	176	ESSKVLNTNGTSGFLPGGYTCFPHSQPWQAALLVQGRLL CGGVLVHPKWVLTAA<u>H</u>CLKE	+	II	-	H	C
3	25,460	25,728	269	GLKVYLGKHALGRVEAGEQVREVVHSIPHPEYRRSPTHL NHDH<u>D</u>IMLLELQSPVQLTGYIQTLPLSHNNRLTPGTTCRV SGWGTTTSPQ	+	I	-	D	A,B,C,D
4	26,879	27,015	137	VNYPKTLQCANIQLRSDEECRQVYPGKITDNMLCAGTKE GGKDSCE	+	0	-	-	A,B,C,D
5	28,778	28,963	189	GD<u>S</u>GGPLVCNRTLYGIVSWGDFPCGQPDRPGVYTRVSRY VLWIRETIRKYETQQQKWLKGPQ	+	-	+	S	A,B,C

* All footnotes same as Table 1

TABLE 5. Predicted exons of the putative gene KLK-L5. The translated protein sequences of each exon (open reading frame) are shown*

Exon No.[1]	Putative coding region[2] From(bp)	To(bp)	No. of bases	Translated protein sequence	EST match[3]	Intron phase[4]	Stop codon[5]	Catalytic triad[6]	Exon prediction program[7]
2	1588	1747	160	LSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGV LIDHRWVL TAAHCSG	-	II	-	H	A,B,C
3	3592	3851	260	SRYWVRLGEHSLSQLDWTEQIRHSGFSVTHPGYLGAS TSHEHDLRLLRLRLPVRVTSSVQPLPLPNDCATAGTEC HVSGWGITNHPR	+	I	-	D	A,B,C,D
4	4806	4939	134	NPFPDLLQCLNLSIVSHATCHGVYPGRITSNMVCAGG VPGQDACQ	+	0	-	-	A,B,C,D

* All footnotes same as Table 1

TABLE 6. Homology between the predicted amino acid sequences of the newly identified putative genes and protein sequences deposited in Genbank

No.	Gene identity	Homolgous known protein	Identity% (number of amino acids)
1	KLK-L1	• Human stratum corneum chymotryptic enzyme	44(101/227)
		• Rat kallikrein	40(96/237)
		• Mouse glandular kallikrein K22	39(94/236)
		• Human glandular kallikrein	38(93/241)
		• Human prostatic specific antigen	37(91/241)
		• Human protease M	37(87/229)
2	KLK-L2	• Human neuropsin	48(106/219)
		• Human stratum corneum chymotryptic enzyme	47(103/216)
		• Human protease M	45(99/219)
		• Human trypsinogen I	45(100/221)
		• Rat trypsinogen	44(98/220)
3	KLK-L3	• Human neuropsin	44(109/244)
		• Rat trypsinogen 4	39(95/241)
		• Human protease M	38(98/253)
		• Human glandular kallikrein	37(94/248)
		• Human prostatic specific antigen	36(89/242)
4	KLK-L4	• Human protease M	52(118/225)
		• Human neuropsin	51(116/225)
		• Mouse neuropsin	51(116/226)
		• Human glandular kallikrein	48(113/234)
		• Human prostatic specific antigen	47(108/227)
5	KLK-L5	• Human neuropsin	44(81/184)
		• Rat trypsinogen I	42(76/178)
		• Rat trypsinogen II	42(75/178)
		• Human protease M	41(73/178)

Table 7. Expressed sequence tags with >95% homology to exons of the prostase/KLK-L1 gene.

GenBank #	**Source**	Tissue	**homologous exons**
AA551449	I.M.A.G.E.	prostate	3,4,5
AA533140	I.M.A.G.E.	prostate	4,5
AA503963	I.M.A.G.E.	prostate	5
AA569484	I.M.A.G.E.	prostate	5
AA336074	TIGR	endometrium	2,3

Table 8. Primers used for reverse transcription-polymerase chain reaction (RT-PCR) analysis of various genes.

Gene	Primer name	Sequence[1]	Product size (base pairs)
Prostase	RS	TGACCCGCTGTACCACCCCA	278
(KLK-L1)	RAS	GAATTCCTTCCGCAGGATGT	
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT	328
	PS2AS	AGGTGTCCGGTGGAGGTGGCA	
PSA	PSAS	TGCGCAAGTTCACCCTCA	754
	PSAAS	CCCTCTCCTTACTTCATCC	
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT	372
	ACTINAS	TCTCCTTAATGTCACGCACGA	

1. All nucleotide sequences are given in the 5'→3' orientation.

Table 9. Tissue expression of prostase/KLK-L1 by RT-PCR analysis

Expression level			
High	**medium**	**low**	**No Expression**
Prostate	Mammary gland	Salivary glands	Stomach
Testis	Colon	Lung	Heart
Adrenals	Spinal cord	Brain	Spleen
Uterus		Bone marrow	Placenta
Thyroid		Thymus	Liver
		Trachea	Pancreas
		Cerebellum	Kidney
			Fetal brain
			Fetal liver
			Skeletal muscle
			Small intestine

Table 10. EST clones with >95% homology to exons of KLK-L2

GENBANK #	Tissue of Origin	I.M.A.GE. ID	Homologous exons
W73140	Fetal heart	344588	4,5
W73168	Fetal heart	344588	3,4,5
AA862032	Squamous cell carcinoma	1485736	4,5
AI002163	Testis	1619481	3,4,5
N80762	Fetal lung	300611	5
W68361	Fetal heart	342591	5
W68496	Fetal heart	342591	5
AA292366	Ovarian tumor	725905	1,2
AA394040	Ovarian tumor	726001	5

Table 11. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence[1]	Product size (base pairs)
KLK-L2	KS	GGATGCTTACCCGAGACAGA	342
	KAS	GCTGGAGAGATGAACATTCT	
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT	328
	PS2AS	AGGTGTCCGGTGGAGGTGGCA	
PSA	PSAS	TGCGCAAGTTCACCCTCA	754
	PSAAS	CCCTCTCCTTACTTCATCC	
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT	372
	ACTINAS	TCTCCTTAATGTCACGCACGA	
KLK-L2	R1	CCGAGACGGACTCTGAAAACTTTCTTCC	
	R2	TGAAAACTTTCTTCCTGCAGTGGGCGGC	

1. All nucleotide sequence are given in the 5' 3' orientation.

Table 12. Tissue expression of KLK-L2 by RT-PCR analysis.

sion level			
high	**Medium**	**low**	**No Expression**
Brain	Salivary gland	Uterus	Stomach
Mammary gland	Fetal brain	Lung	Adrenal gland
Testis	Thymus	Heart	Colon
	Prostate	Fetal liver	Skeletal muscle
	Thyroid	Spleen	
	Trachea	Placenta	
	Cerebellum	Liver	
	Spinal cord	Pancreas	
		Small intestine	
		Kidney	
		Bone marrow	

TABLE 13 . Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence[1]
KLK-L3	L3-F1	CATGCAGTGTCTCATCTCAG
	L3-F2	CATGGAGGAGGAAGGAGATG
	L3-R1	CTTCGGCCTCTCTTGGTCTT
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5' →3' orientation.

TABLE 14. Primers used for gene-specific PCR amplification of the kallikrein genes using DNA as a template.

Primer name	Sequence[1]	Coordinates	GenBank accession #	Gene name
Z1S	GACCCTGACATGTGACATCTA	979-999	U62801	Zyme
Z1AS	GCCACTGCCTGATGGAGACTG	1422-1402		
GL3-F1	AACATCAGCATCCTGGAGAA	7324-7343	AF135026	KLK-L3
LL3-R1	CTTCGGCCTCTCTTGGTCTT	8051-8060		
L2-1	GGGTCAGAGCTGCAGAGAAG	11104-11123	AF135028	KLK-L2
L2-2	GGGCCTGTCGTCTGCAATGG	11522-11541		
KLK-L1	ATGGCCACAGCAGGAAATCC	1411-1430	AF113141	KLK-L1
	GGTCACTTGTCTGCGCAGAC	1990-2019		
PS	CCCAACCCTGTGTTTTTCTC	3634-3653	M33105	PSA[2]
PAS	GGCCCTCCTCCCTCAGA	4143-4118		
K1S	ATCCCTCCATTCCCATCTTT	2-22	M18157	KLK1[3]
K1AS	CACATACAATTCTCTGGTTC	324-30		
K2S	AGTGACACTGTCTCAGAATT	131-150	AF024605	KLK2[4]
K2AS	CCCCAATCTCACCAGTGCAC	580-561		
NS	GCTTCCCTACCGCTGTGCT	552-570	AF055481	NES1[5]
NAS	CACTCTGGCAAGGGTCCTG	763-744		

1. all nucleotide sequences are given in the 5'→ 3' orientation
2. prostate specific antigen
3. human renal kallikrein
4. human glandular kallikrein
5. normal epithelia cell-specific 1 gene.

TABLE 15. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence[1]
KLK-L4	L4-F1	AACTCTACAATGTGCCAACA
	L4-R1	TTATTGTGGGCCCTTCAACC
	L4-R3	GGATGGTCCATTTATAGGAC
	L4-A	AGGCTGCCCTACTAGTGCAA
	L4-B	ATATTGCCTAGGTGGATGTG
	L4-D	AAGACTTCAAGGAGCCAAGC
	L4-E	GACCCTTCACCTCCCAAAAT
	L4-X1	CTAGTGATCGCCTCCCTGAC
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT
	PS2AS	AGGTGTCCGGTGGAGGTGGCA
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5'→3' orientation.

TABLE 16. EST clones with >95% homology to exons of KLK-L4

GenBank #	Tissue of origin	I.M.A.GE. ID
AA399955 AA401397	Testis	743113
AA846771	Testis	1392889
AI002101	Testis	1619045
AI032327	Testis	1644236

TABLE 17. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence[1]
KLK-L5	KLK-L5-F1	TCAGCCAGGCAGCCACACCG
	KLK-L5-R1	TTGGTGATGCCCCAGCCTGA
	L5-F2	CCACACCGAAGATTTTCAAT
	L5-R2	GCCCCTCCTTCATTTATA
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5'→ 3' orientation.

We Claim:

1. An isolated KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 nucleic acid molecule of at least 30 nucleotides which hybridizes to SEQ ID NO. 1, 13, 21, 43, 56, or 65, respectively, or the complement of SEQ ID NO. 1, 13, 21, 43, 56, or 65, under stringent hybridization conditions
2. An isolated nucleic acid molecule which comprises:
   (i) a nucleic acid sequence encoding a protein having substantial sequence identity with an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
   (ii) a nucleic acid sequence encoding a protein comprising an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
   (iii) nucleic acid sequences complementary to (i);
   (iv) a degenerate form of a nucleic acid sequence of (i);
   (v) a nucleic acid sequence capable of hybridizing under stringent conditions to a nucleic acid sequence in (i), (ii) or (iii);
   (vi) a nucleic acid sequence encoding a truncation, an analog, an allelic or species variation of a protein comprising an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively; or
   (vii) a fragment, or allelic or species variation of (i), (ii) or (iii).
3. A purified and isolated nucleic acid molecule of the invention comprises:
   (i) a nucleic acid sequence comprising the sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65 wherein T can also be U;
   (ii) nucleic acid sequences complementary to (i), preferably complementary to the full nucleic acid sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65;
   (iii) a nucleic acid capable of hybridizing under stringent conditions to a nucleic acid of (i) or (ii) and preferably having at least 18 nucleotides; or
   (iv) a nucleic acid molecule differing from any of the nucleic acids of (i) to (iii) in codon sequences due to the degeneracy of the genetic code.
4. An isolated nucleic acid molecule which encodes a protein which binds an antibody of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein.
5. A regulatory sequence of an isolated nucleic acid molecule as claimed in any of the preceding claims fused to a nucleic acid which encodes a heterologous protein.
6. A vector comprising a nucleic acid molecule of any of the preceding claims.
7. A host cell comprising a nucleic acid molecule of any of the preceding claims.

8. An isolated KLK-L1 protein comprising an amino acid sequence of SEQ. ID. NO. 2 or 3.
9. An isolated KLK-L2 protein comprising an amino acid sequence of SEQ. ID. NO. 14.
10. An isolated KLK-L3 protein comprising an amino acid sequence of SEQ. ID. NO. 22 or 23.
11. An isolated KLK-L4 protein comprising an amino acid sequence of SEQ. ID. NO. 44 or 45.
12. An isolated KLK-L5 protein comprising an amino acid sequence of SEQ. ID. NO. 57, 58, 59, or 60.
13. An isolated KLK-L6 protein comprising an amino acid sequence of SEQ. ID. NO. 66 or 67.
14. An isolated protein having at least 65% amino acid sequence identity to an amino acid sequence of SEQ. ID. NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.
15. A method for preparing a protein as claimed in any of the preceding claimd comprising:
    (a) transferring a vector as claimed in claim 6 into a host cell;
    (b) selecting transformed host cells from untransformed host cells;
    (d) culturing a selected transformed host cell under conditions which allow expression of the protein; and
    (e) isolating the protein.
16. A protein prepared in accordance with the method of claim 15.
17. An antibody having specificity against an epitope of a polypeptide as claimed in claim 8, 9, 10, 11, 12, or 13.
18. An antibody as claimed in claim 17 labeled with a detectable substance and used to detect the protein in biological samples, tissues, and cells.
19. A probe comprising a sequence encoding a protein as claimed in claim 8, 9, 10, 11, 12, or 13, or a part thereof.
20. A method of diagnosing and monitoring conditions mediated by a protein as claimed in claim 8, 9, 10, 11, 12, or 13, by determining the presence of a nucleic acid molecule encoding the protein as claimed in any of the preceding claims or determining the presence of the protein.
21. A method as claimed in claim 20 wherein the condition is cancer.
22. A method for identifying a substance which associates with a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising (a) reacting the protein with at least one substance which potentially can associate with the protein, under conditions which permit the association between the substance and protein, and (b) removing or detecting protein associated with the substance, wherein detection of associated protein and substance indicates the substance associates with the prtoein.
23. A method for evaluating a compound for its ability to modulate the biological activity of a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising providing a known concentration of the protein with a substance which associates with the protein and a test compound under conditions which permit the formation of complexes between the substance and protein, and removing and/or detecting complexes.
24. A method for detecting a nucleic acid molecule encoding a protein comprising an amino acid sequence of SEQ. ID. NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67 in a biological sample comprising the steps of:

(a) hybridizing a nucleic acid molecule of claim 2 to nucleic acids of the biological sample, thereby forming a hybridization complex; and

(b) detecting the hybridization complex wherein the presence of the hybridization complex correlates with the presence of a nucleic acid molecule encoding the protein in the biological sample.

25. A method as claimed in claim 24 wherein nucleic acids of the biological sample are amplified by the polymerase chain reaction prior to the hybridizing step.

26. A method for treating a condition mediated by a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising administering an effective amount of an antibody as claimed in claim 17 or a substance or compound identified in accordance with a method claimed in claim 22 or 23.

27. A method as claimed in claim 26 wherein the condition is cancer.

28. A composition comprising one or more of a nucleic acid molecule or protein claimed in any of the preceding claims, or a substance or compound identified using a method as claimed in any of the preceding claims, and a pharmaceutically acceptable carrier, excipient or diluent.

29. Use of one or more of a nucleic acid molecule or protein claimed in any of the preceding claims, or a substance or compound identified using a method as claimed in any of the preceding claims in the preparation of a pharmaceutical composition for treating a condition mediated by a protein as claimed in any of the preceding claims.

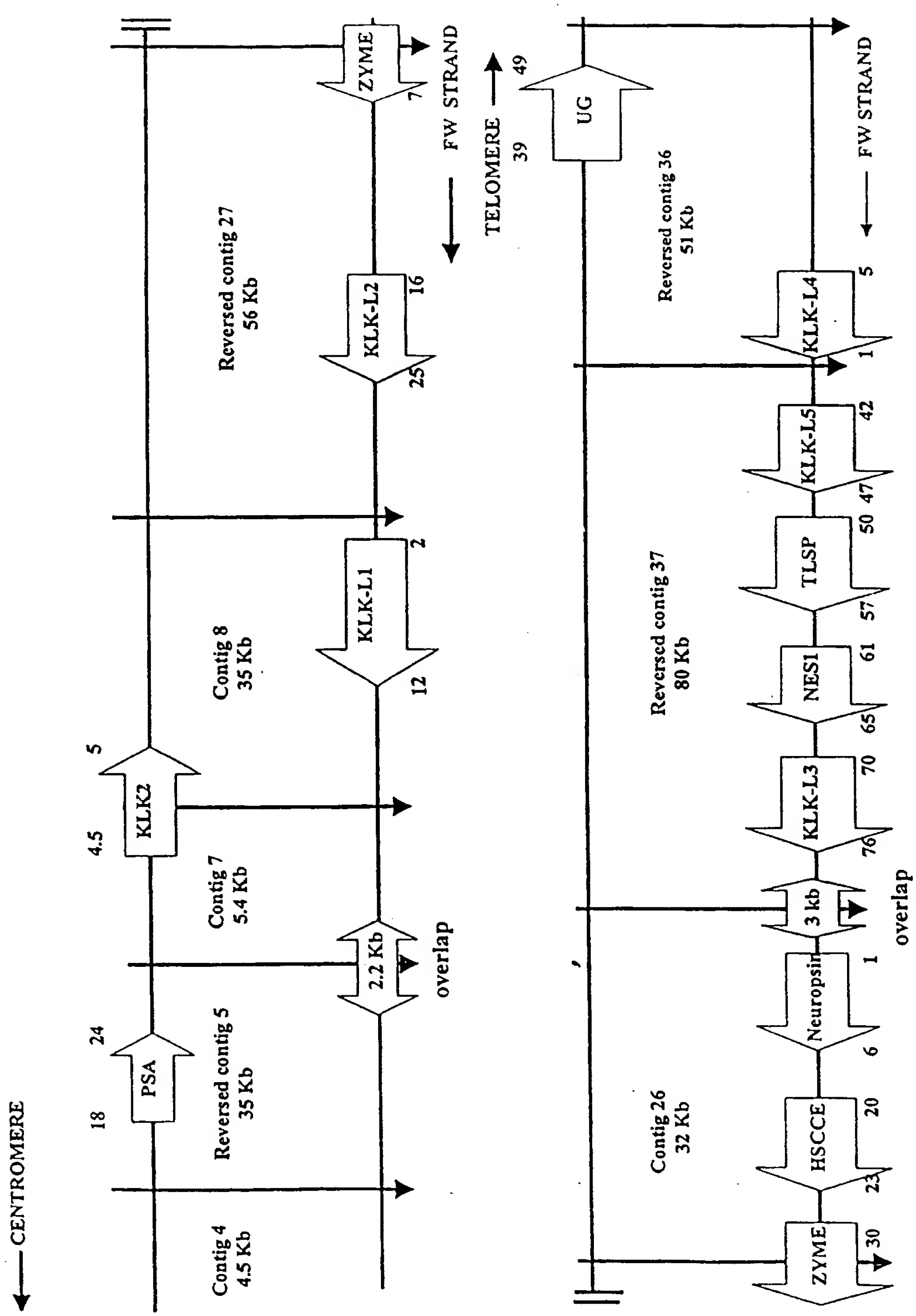
30. A transgenic non-human mammal which doe not express a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as claimed in claim 8, 9, 10, 11, 12, or 13, respectively, resulting in a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology, respectively.

31. A transgenic animal assay system which provides a model system for testing for an agent that reduces or inhibits an a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology comprising

(a) administering the agent to a transgenic non-human animal as claimed in claim 26; and

(b) determining whether said agent reduces or inhibits a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology in the transgenic non-human animal relative to a transgenic non-human animal of step (a) which has not been administered the agent.

FIGURE 1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TELOMERE
FW STRAND
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4.5 Kb
Reversed contig 5
35 Kb
PSA
18
24
2.2 Kb
overlap
Contig 7
5.4 Kb
KLK2
4.5
5
Contig 8
35 Kb
KLK-L1
2
12
Reversed contig 27
56 Kb
KLK-L2
16
25
ZYME
7
UG
39
49
Reversed contig 36
51 Kb
KLK-L4
5
1
Reversed contig 37
80 Kb
KLK-L5
42
47
TLSP
50
57
NES1
61
65
KLK-L3
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76
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overlap
Neuropsin
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6
Contig 26
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HSCCE
20
23
ZYME
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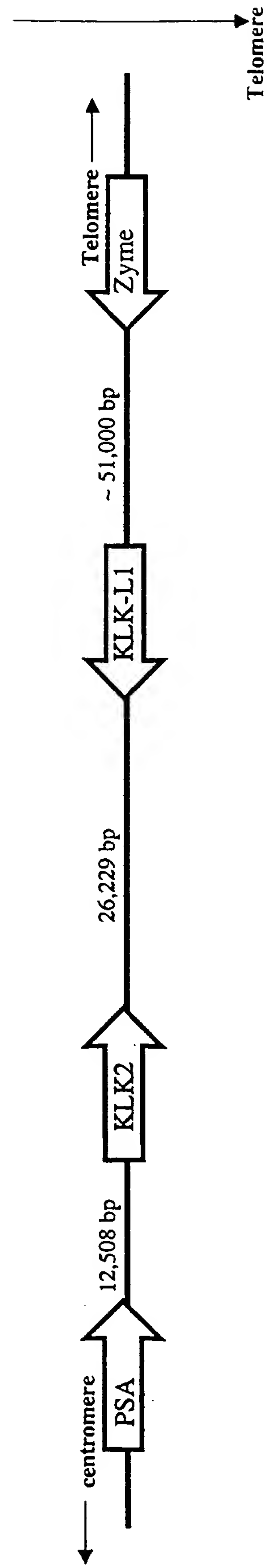
Telomere
Telomere
Zyme
~ 51,000 bp
KLK-L1
26,229 bp
KLK2
12,508 bp
PSA
centromere

FIGURE 2

FIGURE 3

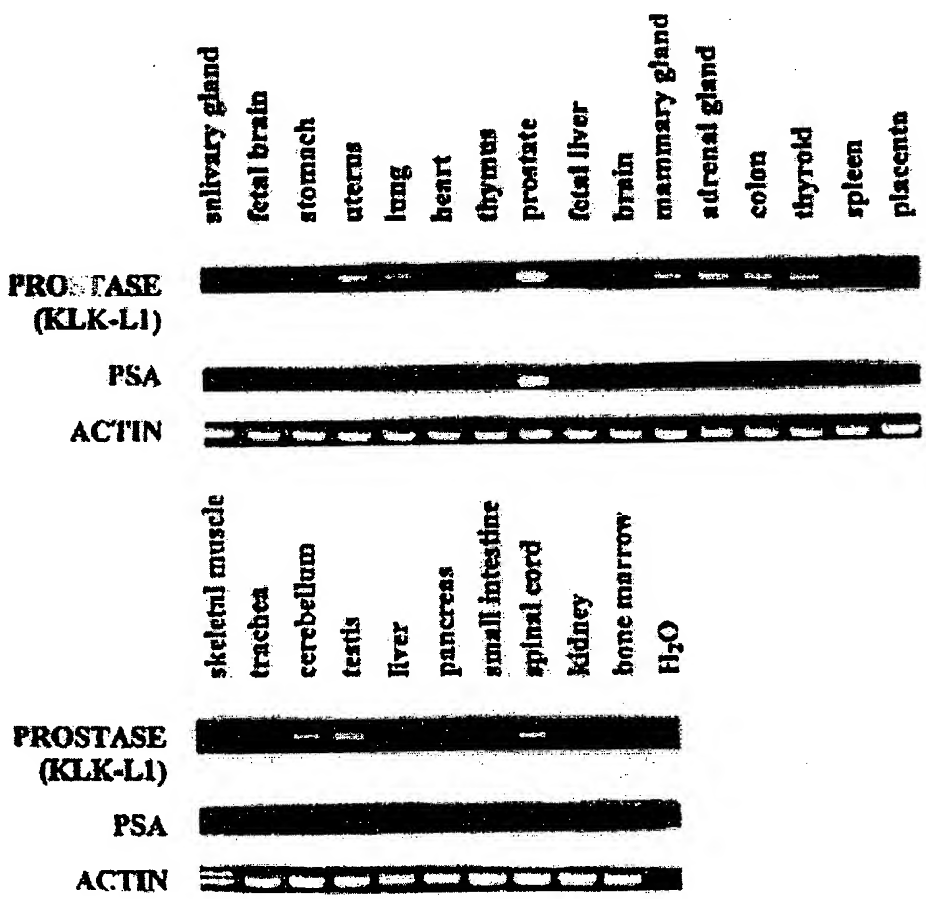
salivary gland
fetal brain
stomach
uterus
lung
heart
thymus
prostate
fetal liver
brain
mammary gland
adrenal gland
colon
thyroid
spleen
placenta
PROSTASE (KLK-L1)
PSA
ACTIN
skeletal muscle
trachea
cerebellum
testis
liver
pancreas
small intestine
spinal cord
kidney
bone marrow
H_2O
PROSTASE (KLK-L1)
PSA
ACTIN

FIGURE 4

TGACCCGCTG TACCACCCCA GCATGTTCTG CGCCGGCGGA GGGCAAGACC
AGAAGGACTC CTGCAACGGT GACTCTGGGG GGCCCCTGAT CTGCAACGGG
TACTTGCAGG GCCTTGTGTC TTTCGGAAAA GCCCCGTGTG GCCAAGTTGG
CGTGCCAGGT GCCTACACCA ACCTCTGCAA ATTCACTGAG TGGATAGAGA
AAACCGTCCA GGCCAGTTAA CTCTGGGGAC TGGGAACCCA TGAAATTGAC
CCCCAAATAC ATCCTGCGGA AGGAATTC

FIGURE 5

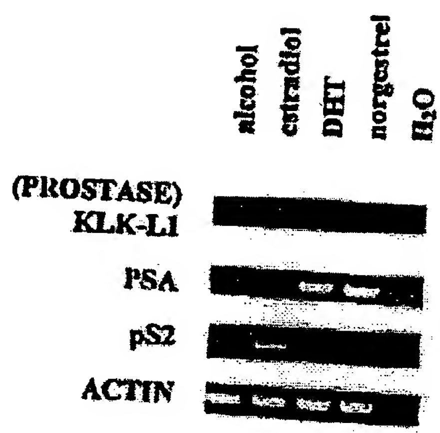
alcohol
estradiol
DHT
norgestrel
H_2O
(PROSTASE)
KLK-L1
PSA
pS2
ACTIN

FIGURE 6

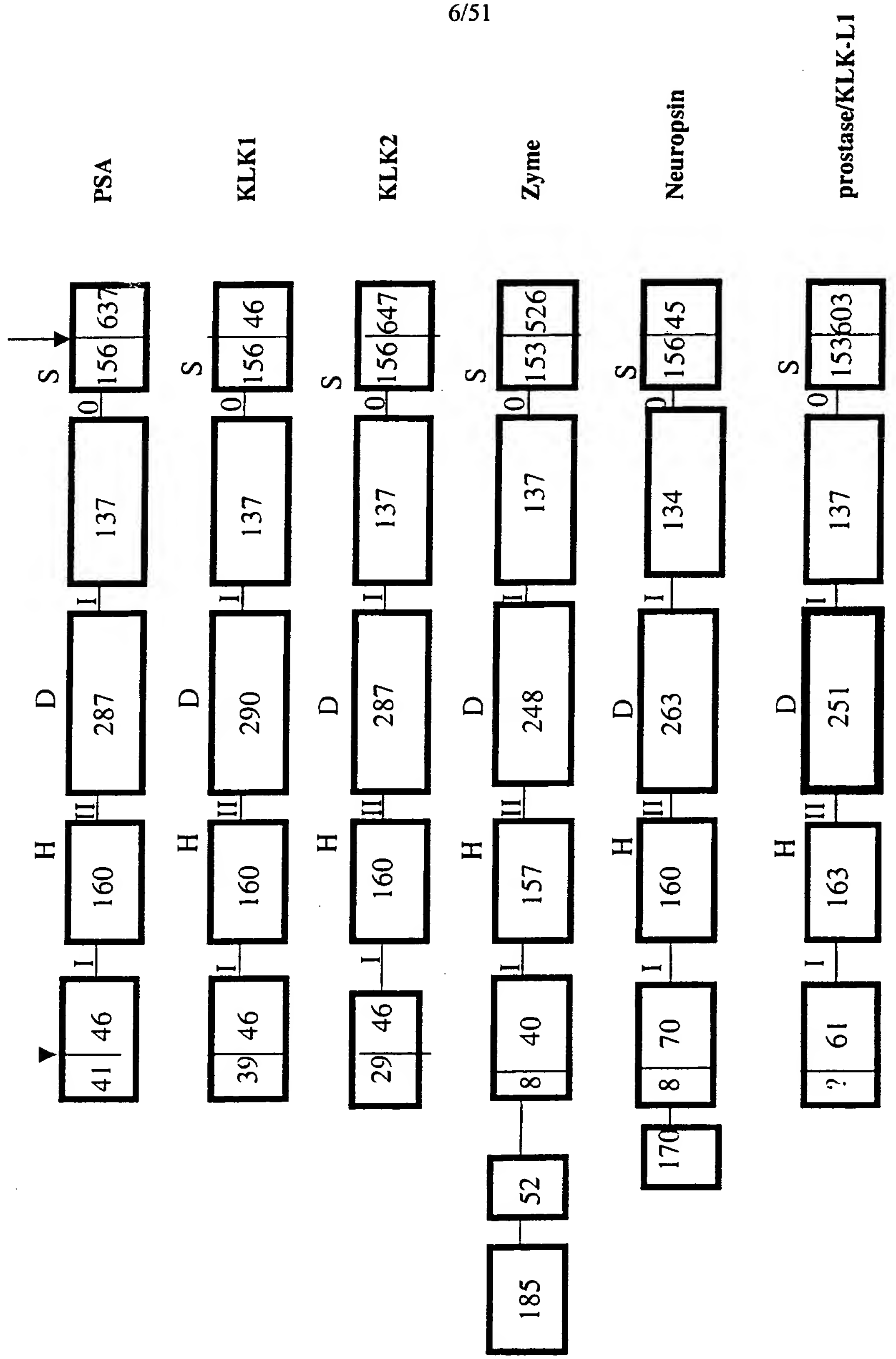
PSA
KLK1
KLK2
Zyme
Neuropsin
prostase/KLK-L1
S
D
H
I
II
III
0
41
46
160
287
137
156
637
39
290
29
647
8
40
157
248
153
526
52
185
70
263
134
45
170
?
61
163
251
603

FIGURE 7

ATGGCTACAGCAAGACCCCCCTGGATGTGGGTGCTCTGTGCTCTGATCACAGCCT
M A T A R P P W M W V L C A L I T A
TGCTTCTGGGGGTCACAGgtaaccaga ---------------------- intron 1--------------------tcccag
L L L G V T
AGCATGTTCTCGCCAACAATGATGTTTCCTGTGACCACCCCTCTAACACCGTGCCC
E H V L A N N D V S C D H P S N T V P
TCTGGGAGCAACCAGGACCTGGGAGCTGGGGCCGGGGAAGACGCCCGGTCGGAT
S G S N Q D L G A G A G E D A R S D
GACAGCAGCAGCCGCATCATCAATGGATCCGACTGCGATATGCACACCCAGCCGT
D S S S R I I N G S D C D M H T Q P
GGCAGGCCGCGCTGTTGCTAAGGCCCAACCAGCTCTACTGCGGGGCGGTGTTGGT
W Q A A L L L R P N Q L Y C G A V L V
GCATCCACAGTGGCTGCTCACGGCCGCCCACTGCAGGAAGAAgtgagtggga------------
H P Q W L L T A A H C R K K
------------------------ intron 2----------------------------tcttcctcagAGTTTTCAGAGTCCGTCT
V F R V R L
CGGCCACTACTCCCTGTCACCAGTTTATGAATCTGGGCAGCAGATGTTCCAGGGG
G H Y S L S P V Y E S G Q Q M F Q G
GTCAAATCCATCCCCCACCCTGGCTACTCCCACCCTGGCCACTCTAACGACCTCAT
V K S I P H P G Y S H P G H S N D L M
GCTCATCAAACTGAACAGAAGAATTCGTCCCACTAAAGATGTCAGACCCATCAAC
L I K L N R R I R P T K D V R P I N
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V S S H C P S A G T K C L V S G W G T
AACCAAGAGCCCCCAAGgtgagtgtccaggt ------------------------intron 3--------- -----tgacag
T K S P Q
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V H F P K V L Q C L N I S V L S Q K R
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C E D A Y P R Q I D D T M F C A G D
AAAGCAGGTAGAGACTCCTGCCAGgtgaggacacc--------------- intron 4------------------
ag
K A G R D S C Q
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G D S G G P V V C N G S L Q G L V S
GGGGAGATTACCCTTGTGCCCGGCCCAACAGACCGGGTGTCTACACGAACCTCTG
W G D Y P C A R P N R P G V Y T N L C
CAAGTTCACCAAGTGGATCCAGGAAACCATCCAGGCCAACTCCTGAGTCATCC
CA
K F T K W I Q E T I Q A N S
GGACTCAGCACACCGGCATCCCCACCTGCTGCAGGGACAGCCCTGACACTCCTTT
CAGACCCTCATTCCTTCCCAGAGATGTTGAGAATGTTCATCTCTCCAGCCCCTGAC
CCCATGTCTCCTGGACTCAGGGTCTGCTTCCCCCACATTGGGCTGACCGTGTCTCT
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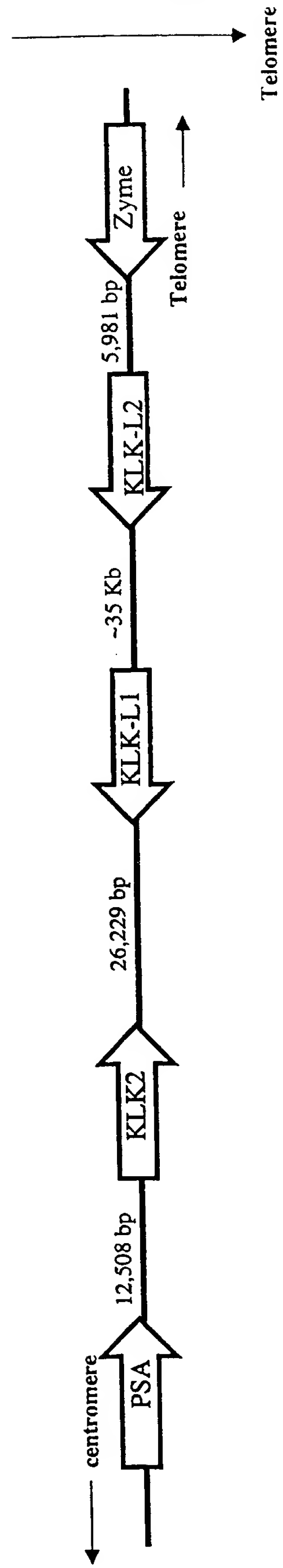

Telomere
Zyme
Telomere
5,981 bp
KLK-L2
~35 Kb
KLK-L1
26,229 bp
KLK2
12,508 bp
PSA
centromere

FIGURE 8

FIGURE 9

```
prostase     MATAGNPWGWFLG----YLILGVAGSLVSG------------------------------ 26
EMSP         MATAGNPWGWFLG----YLILGVAGSLVSG------------------------------ 26
KLK-L2       MATARPPWMWVLCALITALLLGVTEHVLANNDVSCDHPSNTVPSGSNQDLGAGAGEDARS 60
zyme         --------MKKLM-----VVLSLIAAAWA------------------------------- 16
neuropsin    -MGRPRPRAAKTW-----MFLLLLGGAWAGH---------------------------S 26
TLSP         --------MRILQ-----LILLALATGLVG------------------------------ 17
PSA          ------MWVPVVF-----LTLSVTWIGAAPL----------------------------- 20
KLK2         ------MWDLVLS-----IALSVGCTGAVPL----------------------------- 20
KLK1         ------MWFLVLC-----LALSLGGTGAAPP----------------------------- 20
trypsinogen  -------MNPLLI-----LTFVAAALAAPFD----------------------------- 19

prostase     --SCSQIINGEDCSPHSQPWQAALVM-ENELFCSGVLVHPQWVLSAAHCFQNSYTIGLGL 83
EMSP         --SCSQIINGEDCSPHSQPWQAALVM-ENELFCSGVLVHPQWVLSAAHCFQNSYTIGLGL 83
KLK-L2       DDSSSRIINGSDCDMHTQPWQAALLLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVRLGH 120
zyme         -EEQNKLVHGGPCDKTSHPYQAALYT-SGHLLCGGVLIHPLWVLTAAHCKKPNLQVFLGK 74
neuropsin    RAQEDKVLGGHECQPHSQPWQAALFQ-GQQLLCGGVLVGGNWVLTAAHCKKPKYTVRLGD 85
TLSP         --GETRIIKGFECKPHSQPWQAALFE-KTRLLCGATLIAPRWLLTAAHCLKPRYIVHLGQ 74
PSA          --ILSRIVGGWECEKHSQPWQVLVAS-RGRAVCGGVLVHPQWVLTAAHCIRNKSVILLGR 77
KLK2         --IQSRIVGGWECEKHSQPWQVAVYS-HGWAHCGGVLVHPQWVLTAAHCLKKNSQVWLGR 77
KLK1         --IQSRIVGGWECEQHSQPWQAALYH-FSTFQCGGILVHRQWVLTAAHCISDNYQLWLGR 77
trypsinogen  --DDDKIVGGYNCEENSVPYQVSLNS--GYHFCGGSLINEQWVVSAGHCYKSRIQVRLGE 75

prostase     HSLEADQEPGSQMVEASLSVRHPEYN----RP-------LLANDLMLIKLDESVS-ESDT 131
EMSP         HSLEADQEPGSQMVEASLSVRHPEYN----RP-------LLANDLMLIKLDESVS-ESDT 131
KLK-L2       YSLSPVYESGQQMFQGVKSIPHPGYS----HP-------GHSNDLMLIKLNRRIR-PTKD 168
zyme         HNLRQ-RESSQEQSSVVRAVIHPDY----DAA-------SHDQDIMLLRLARPAK-LSEL 121
neuropsin    HSLQN-KDGPEQEIPVVQSIPHPCYN-SSDVE-------DHNHDLMLLQLRDQAS-LGSK 135
TLSP         HNLQK-EEGCEQTRTATESFPHPGFNNSLPNK-------DHRNDIMLVKMASPVS-ITWA 125
PSA          HSLFH-PEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHDLMLLRLSEPAE-LTDA 135
KLK2         HNLFE-PEDTGQRVPVSHSFPHPLYNMSLLKHQSLRPDEDSSHDLMLLRLSEPAK-ITDV 135
KLK1         HNLFD-DENTAQFVHVSESFPHPGFNMSLLENHTRQADEDYSHDLMLLRLTEPADTITDA 136
trypsinogen  HNIEV-LEGNEQFINAAKIIRHPQYDRKTLNN-----------DIMLIKLSSRAV-INAR 122

prostase     IRSISIASQCPTAGNSCLVSGWGLLANG--RMPTVLQCVNVSVVSEEVCSKLYDPLYHPS 189
EMSP         IRSISIASQCPTAGNSCLVSGWGLLANG--RMPTVLQCVNVSVVSEEVCSKLYDPLYHPS 189
KLK-L2       VRPINVSSHCPSAGTKCLVSGWGTTKSPQVHFPKVLQCLNISVLSQKRCEDAYPRQIDDT 228
zyme         IQPLPLERDCSANTTSCHILGWGKTADG--DFPDTIQCAYIHLVSREECEHAYPGQITQN 179
neuropsin    VKPISLADHCTQPGQKCTVSGWGTVTSPRENFPDTLNCAEVKIFPQKKCEDAYPGQITDG 195
TLSP         VRPLTLSSRCVTAGTSCLISGWGSTSSPQLRLPHTLRCANITIIEHQKCENAYPGNITDT 185
PSA          VKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKF 195
KLK2         VKVLGLPTQEPALGTTCYASGWGSIEPEEFLRPRSLQCVSLHLLSNDMCARAYSEKVTEF 195
KLK1         VKVVELPTEEPEVGSTCLASGWGSIEPENFSFPDDLQCVDLKILPNDECKKAHVQKVTDF 196
trypsinogen  VSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKCEASYPGKITSN 182

prostase     MFCAGGGHDQKDSCNGDSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEK 249
EMSP         MFCAGGGHDQKDSCNGDSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEK 249
KLK-L2       MFCAG-DKAGRDSCQGDSGGPVVCNGSLQGLVSWGDYPCARPNRPGVYTNLCKFTKWIQE 287
zyme         MLCAGDEKYGKDSCQGDSGGPLVCGDHLRGLVSWGNIPCGSKEKPGVYTNVCRYTNWIQK 239
neuropsin    MVCAGSSK-GADTCQGDSGGPLVCDGALQGITSWGSDPCGRSDKPGVYTNICRYLDWIKK 254
TLSP         MVCASVQEGGKDSCQGDSGGPLVCNQSLQGIISWGQDPCAITRKPGVYTKVCKYVDWIQE 245
PSA          MLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKD 255
KLK2         MLCAGLWTGGKDTCGGDSGGPLVCNGVLQGITSWGPEPCALPEKPAVYTKVVHYRKWIKD 255
KLK1         MLCVGHLEGGKDTCVGDSGGPLMCDGVLQGVTSWGYVPCGTPNKPSVAVRVLSYVKWIED 256
trypsinogen  MFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWG-DGCAQKNKPGVYTKVYNYVKWIKN 241
```

FIGURE 10

(A)

```
            +-----------------------------zyme
     +----5
     !      +----------------------------neuropsin
     !
     !                                           +----------PSA
     !                                   +-------2
     !      +------------------3                 +------KLK2
     !      !                           !
  --8----6                               +---------------KLK1
     !      !
     !      +-----------------------------trypsinogen
     !
     !                                                      +prostase (KLK-
L1)
     !               +-------------------------------1
     !    +---------4                                       +EMSP
     !    !          !
     +--7            +----------------KLK-L2
          !
          +------------------------TLSP
```

(B)

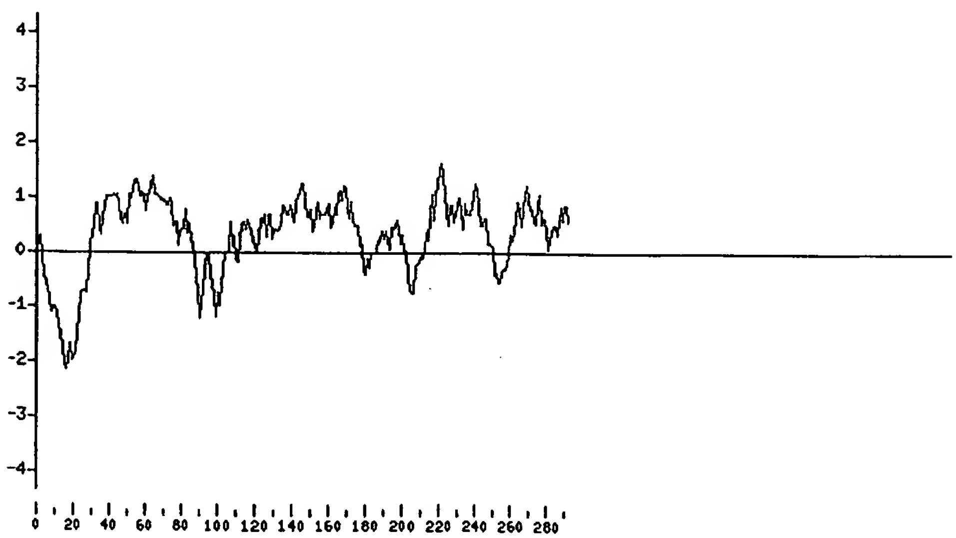


FIGURE 11

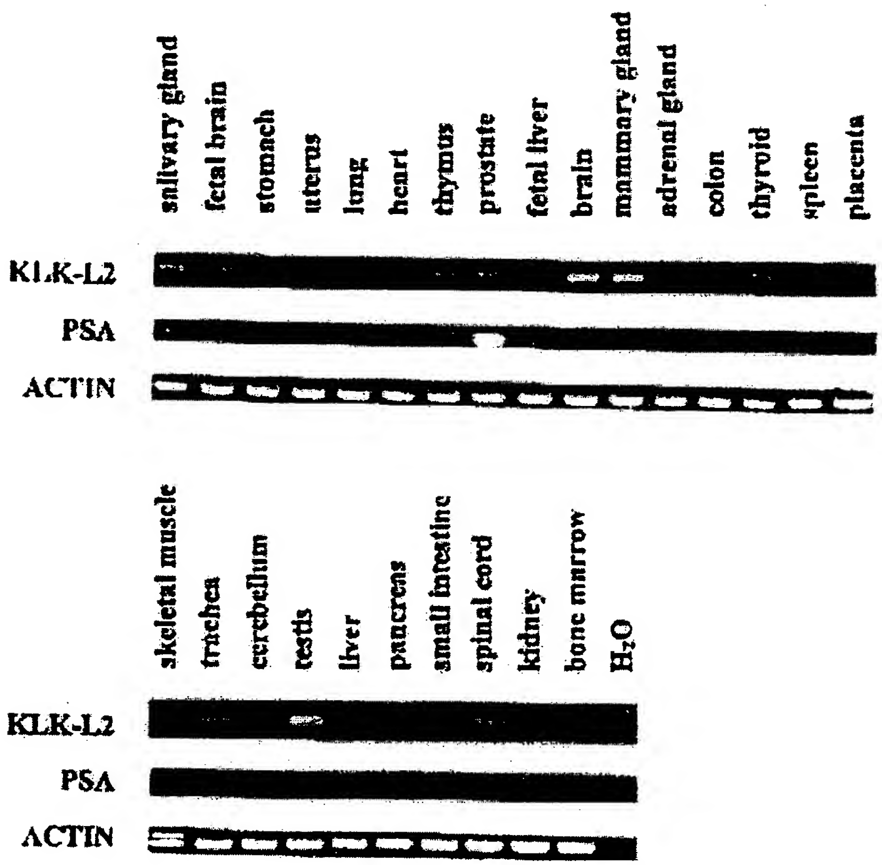

salivary gland
fetal brain
stomach
uterus
lung
heart
thymus
prostate
fetal liver
brain
mammary gland
adrenal gland
colon
thyroid
spleen
placenta
KLK-L2
PSA
ACTIN
skeletal muscle
trachea
cerebellum
testis
liver
pancreas
small intestine
spinal cord
kidney
bone marrow
H2O
KLK-L2
PSA
ACTIN

FIGURE 12

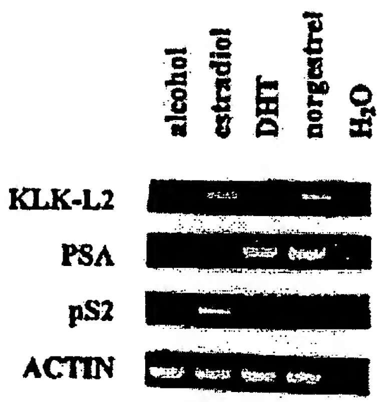


FIGURE 13

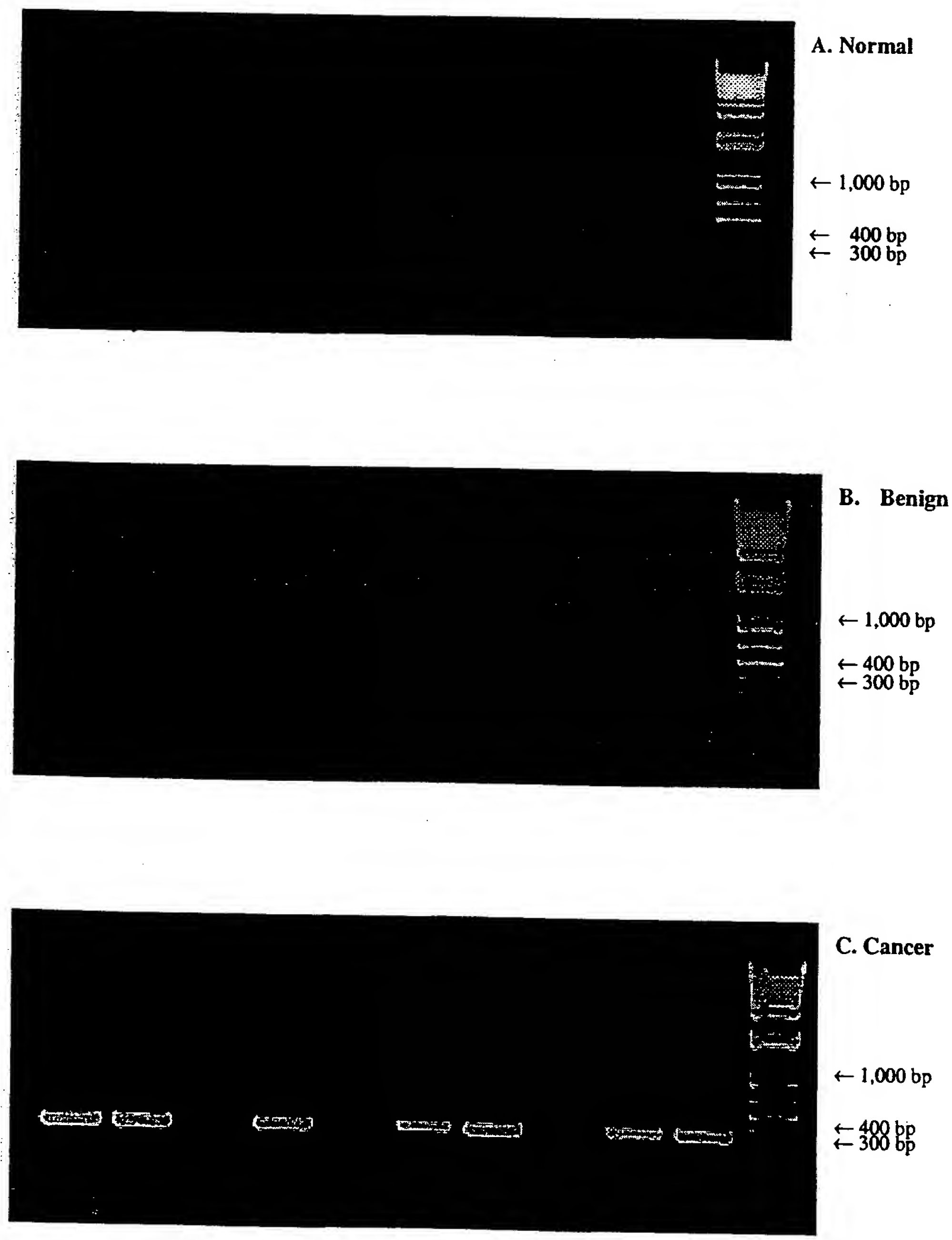

A. Normal
← 1,000 bp
← 400 bp
← 300 bp
B. Benign
← 1,000 bp
← 400 bp
← 300 bp
C. Cancer
← 1,000 bp
← 400 bp
← 300 bp

FIGURE 14

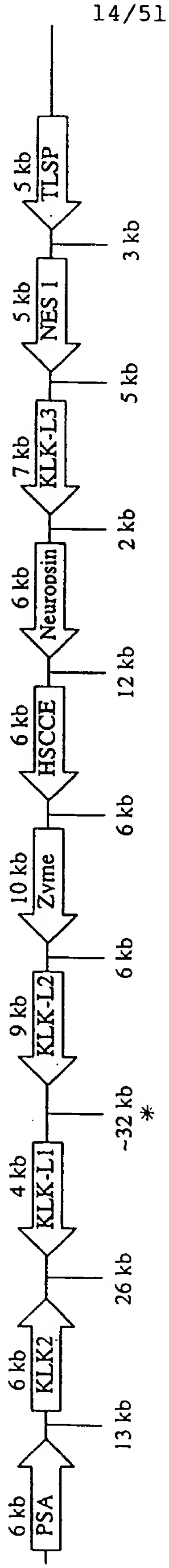

6 kb
PSA
13 kb
6 kb
KLK2
26 kb
4 kb
KLK-L1
~32 kb
*
9 kb
KLK-L2
6 kb
10 kb
Zyme
6 kb
6 kb
HSCCE
12 kb
6 kb
Neuropsin
2 kb
7 kb
KLK-L3
5 kb
5 kb
NES 1
3 kb
5 kb
TLSP

FIGURE 15

CACTGGACGGGTGCACGTTCAGGATCCAGGTGCCCAGGGGTCATGAAG CTG GGA CTC
M K L G L

CTC TGT GCT CTG CTC TCT CTG CTG GCA G gtga... intron 1 ..ctag GG CAT GGC
L C A L L S L L A G H G

TGG GCA GAC ACC CGT GCC ATC GGG GCC GAG GAA TGT CGC CCC AAC TCC CAG
W A D T R A I G A E E C R C N S Q

CCT TGG CAG GCC GGC CTC TTC CAC CTT ACT CGG CTC TTC TGT GGG GCG ACC
P W Q A G L F H L T R L F C G A T

CTC ATC AGT GAC CGC TGG CTG CTC ACA GCT GCC CAC TGC CGC AAG CCgtga......
L I S D R W L L T A A H C R K P

intron 2 ......gcagG TAT CTG TGG GTC CGC CTT GGA GAG CAC CAC CTC TGG AAA
Y L W V R L G E H H L W K

TGG GAG GGT CCG GAG CAG CTG TTC CGG GTT ACG GAC TTC TTC CCC CAC CCT
W E G P E Q L F R V T D F F P H P

GGC TTC AAC AAG GAC CTC AGC GCC AAT GAC CAC AAT GAT GAC ATC ATG CTG
G F N K D L S A N D H N D D I M L

ATC CGC CTG CCC AGG CAG GCA CGT CTG AGT CCT GCT GTG CAG CCC CTC AAC
I R L P R Q A R L S P A V Q P L N

CTC AGC CAG ACC TGT GTC TCC CCA GGC ATG CAG TGT CTC ATC TCA GGC TGG
L S Q T C V S P G M Q C L I S G W

GGG GCC GTG TCC AGC CCC AAG G gtat...... intron acag CG CTG TTT CCA GTC
G A V S S P K A L F P V

ACA CTG CAG TGT GCC AAC ATC AGC ATC CTG GAG AAC AAA CTC TGT CAC TGG
T L Q C A N I S I L E N K L C H W

GCA TAC CCT GGA CAC ATC TCG GAC AGC ATG CTC TGT GCG GGC CTG TGG GAG
A Y P G H I S D S M L C A G L W E

GGG GGC CGA GGT TCC TGC CAG gtga...... intron ..acag GGT GAC TCT GGG GGC
G G R G S C Q G D S G G

CCC CTG GTT TGC AAT GGA ACC TTG GCA GGC GTG GTG TCT GGG GGT GCT GAG
P L V C N G T L A G V V S G G A E

FIGURE 15 (CONT'D)

CCC TGC TCC AGA CCC CGG CGC CCC GCA GTC TAC ACC AGC GTA TGC CAC TAC
P C S R P R R P A V Y T S V C H Y

CTT GAC TGG ATC CAA GAA ATC ATG GAG AAC TGA
L D W I Q E I M E N
GCCCGCGCGCCACGGGGGCACCTTGGAAGACCAAGAGAGGCCGAAGGGCACGGGGTA
GGGGGTTCTCGTAGGGTCCCAGCCTCAATGGTTCCCGCCCTGGACCTCCAGCTGCCCTG
ACTCCCCTCTGGACACTAAGACTCCGCCCCTGAGGCTCCGCCCCCTCACGGGTCAAGCA
AGACACAGTCGCGCCCCCTCGGAACGGAGCAGGGACACGCCCTTCAGAGCCGTCTCTAT
GACGTCACCGACAGCCATCACCTCCTTCTTGGAACAGCACAGCCTGTGGCTCCGCCCCA
AGGAACCACTTACACAAAATAGCTCCGCCCCTCGGAACTTTGCCCAGTGGGACTTCCCC
TCGGGACTCCACCCCTTGTGGCCCCGCCTCCTTCACCAGAGATCTCGCCCCTCGTGATGT
CAGGGGCGCAGTAGCTCCGCCCACGTGGAGCTCGGGCGGTGTAGAGCTCAGCCCTTGTG
GCCCCGTCCTGGGCGTGTGCTGGGTTTGAATCCTGGCGGAGACCTGGGGGGAAATTGAG
GGAGGGTCTGGATACCTTTAGAGCCAATGCAACGGATGATTTTTCAGTAAACGGGGAAA
CCTCA

FIGURE 16

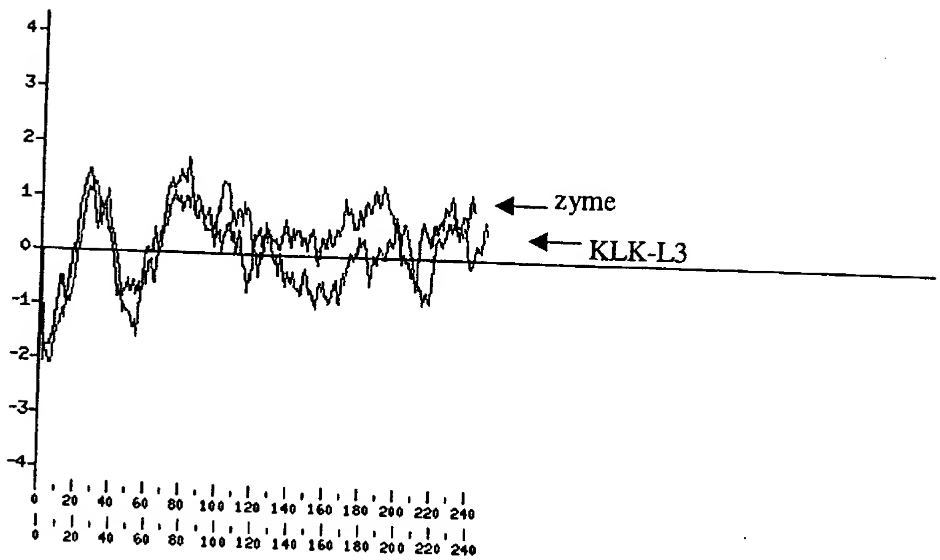

4
3
2
1
0
-1
-2
-3
-4
zyme
KLK-L3
0 20 40 60 80 100 120 140 160 180 200 220 240
0 20 40 60 80 100 120 140 160 180 200 220 240

FIGURE 17

```
PSA          ---------MWVPVVFLTLSVTWIGAAPLI-LSRIVGGWECEKHSQPWQVLVASRGRAVC
KLK2         ---------MWDLVLSIALSVGCTGAVPLI-QSRIVGGWECEKHSQPWQVAVYSHGWAHC
KLK1         ---------MWFLVLCLALSLGGTGAAPPI-QSRIVGGWECEQHSQPWQAALYHFSTFQC
trypsinogen  ----------MNPLLILTFVAAALAAPFDD-DDKIVGGYNCEENSVPYQVSLNS-GYHFC
KLK-L3       ----------MKLGLLCALLSLLAGHGWA--DTRAIGAEECRPNSQPWQAGLFHLTRLFC
TLSP         ----------MRI-LQLILLALATGLVGG--ETRIIKGFECKPHSQPWQAALFEKTRLLC
neuropsin    -MGRPRPRAAKTWMFLLLLGGAWAGHSRAQ-EDKVLGGHECQPHSQPWQAALFQGQQLLC
zyme         ----------MKK--LMVVLSLIAAAWAEE-QNKLVHGGPCDKTSHPYQAALYTSGHLLC
HSCCE        ---MARSLLLPLQILLLSLALETAGEEAQG--DKIIDGAPCARGSHPWQVALLSGNQLHC
prostase     ---MA-TAGNPWGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVMENELFC

PSA          GGVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDT-GQVFQVSHSFPHPLYDMSLLKNR
KLK2         GGVLVHPQWVLTAAHCLKKNSQVWLGRHNLFEPEDT-GQRVPVSHSFPHPLYNMSLLKHQ
KLK1         GGILVHRQWVLTAAHCISDNYQLWLGRHNLFDDENT-AQFVHVSESFPHPGFNMSLLENH
trypsinogen  GGSLINEQWVVSAGHCYKSRIQVRLGEHNIEVLEGN-EQFINAAKIIRHPQYDRKTLNN-
KLK-L3       GATLISDRWLLTAAHCRKPYLWVRLGEHHLWKWEGP-EQLFRVTDFFPHPGFNKDLSAN-
TLSP         GATLIAPRWLLTAAHCLKPRYIVHLGQHNLQKEEGC-EQTRTATESFPHPGFNNSLPNK-
neuropsin    GGVLVGGNWVLTAAHCKKPKYTVRLGDHSLQNKDGP-EQEIPVVQSIPHPCYNSSD-VE-
zyme         GGVLIHPLWVLTAAHCKKPNLQVFLGKHNLRQRESS-QEQSSVVRAVIHPDYDAAS----
HSCCE        GGVLVNERWVLTAAHCKMNEYTVHLGSDTLGDRR---AQRIKASKSFRHPGYSTQT----
prostase     SGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSQMVEASLSVRHPEYNRPLLAN-

PSA          FLRPGDDSSHDLMLLRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP
KLK2         SLRPDEDSSHDLMLLRLSEPAK-ITDVVKVLGLPTQEPALGTTCYASGWGSIEPEEFLRP
KLK1         TRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEEPEVGSTCLASGWGSIEPENFSFP
trypsinogen  ----------DIMLIKLSSRAV-INARVSTISLPTAPPATGTKCLISGWGNTASSGADYP
KLK-L3       ------DHNDDIMLIRLPRQAR-LSPAVQPLNLSQTCVSPGMQCLISGWGAVSSPKALFP
TLSP         ------DHRNDIMLVKMASPVS-ITWAVRPLTLSSRCVTAGTSCLISGWGSTSSPQLRLP
neuropsin    ------DHNHDLMLLQLRDQAS-LGSKVKPISLADHCTQPGQKCTVSGWGTVTSPRENFP
zyme         -------HDQDIMLLRLARPAK-LSELIQPLPLERDCSANTTSCHILGWGKTADG--DFP
HSCCE        -------HVNDLMLVKLNSQAR-LSSMVKKVRLPSRCEPPGTTCTVSGWGTTTSPDVTFP
prostase     ----------DLMLIKLDESVS-ESDTIRSISIASQCPTAGNSCLVSGWGLLANG--RMP

PSA          KKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITS
KLK2         RSLQCVSLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKDTCGGDSGGPLVCNGVLQGITS
KLK1         DDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVGDSGGPLMCDGVLQGVTS
trypsinogen  DELQCLDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVS
KLK-L3       VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQGDSGGPLVCNGTLAGVVS
TLSP         HTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGPLVCNQSLQGIIS
neuropsin    DTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGPLVCDGALQGITS
zyme         DTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCGDHLRGLVS
HSCCE        SDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGPLVCRGTLQGLVS
prostase     TVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGPLICNGYLQGLVS

PSA          WGSEPCALPERPSLYTKVVHYRKWIKDTIVANP
KLK2         WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANP
KLK1         WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENS
trypsinogen  WG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANS
KLK-L3       GGAEPCSRPRRPAVYTSVCHYLDWIQEIMEN--
TLSP         WGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-
neuropsin    WGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKG
zyme         WGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-
HSCCE        WGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR
prostase     FGKAPCGQVGVPGVYTNLCKFTEWIEKTVQAS-
```

FIGURE 18

```
                  +------TLSP
             +----4
             !    +-------------KLK-L3
         +-13
         !   !  +-------------neuropsin
         !   +-12
     +-14       +---------------------NES 1
     !   !
     !   !   +------------zyme
     !   +-11
     !       +-------------KLK-L4
     !
     !                          +-----PSA
     !                     +---2
     !   +----------3      +---KLK2
     !   !              !
     !   !              +--------KLK1
   -15-10
     !   !   +------------trypsinogen I
     !   !   !
     !   !   !                 +----------------complement factor D
     !   +--9             +--6
     !       !    +---7    +--------------------granzyme A
     !       !    !   !
     !       +--8      +--------------------cathepsin G
     !          !
     !          +--------------------chymotrypsin
     !
     !                          +prostase/KLK-L1
     !     +-------------1
     +----5                     +EMSP
           !
           +----------KLK-L2
```

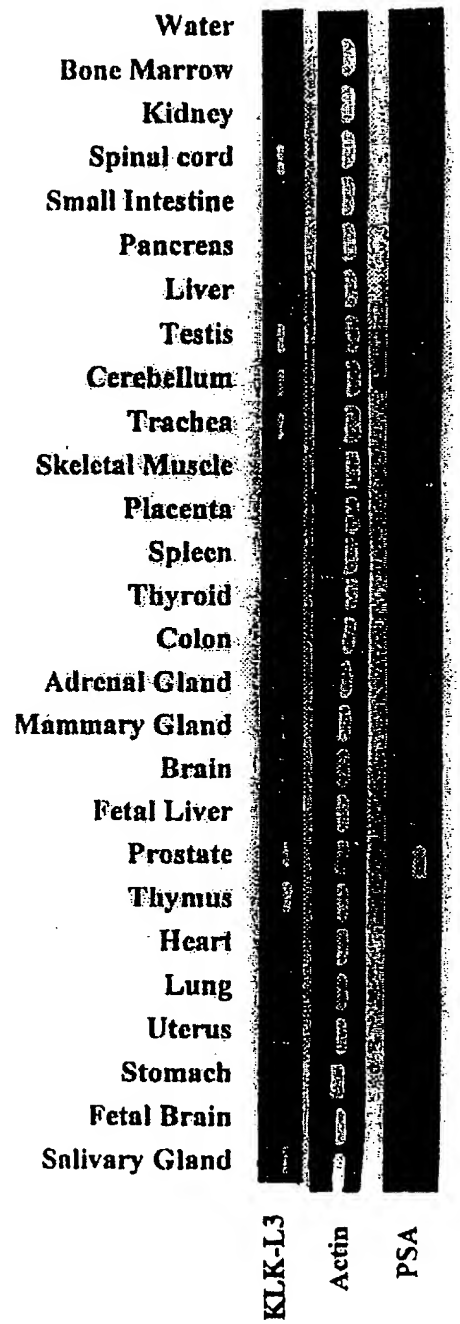

Water
Bone Marrow
Kidney
Spinal cord
Small Intestine
Pancreas
Liver
Testis
Cerebellum
Trachea
Skeletal Muscle
Placenta
Spleen
Thyroid
Colon
Adrenal Gland
Mammary Gland
Brain
Fetal Liver
Prostate
Thymus
Heart
Lung
Uterus
Stomach
Fetal Brain
Salivary Gland
KLK-L3
Actin
PSA

FIGURE 19

FIGURE 20

FIGURE 21

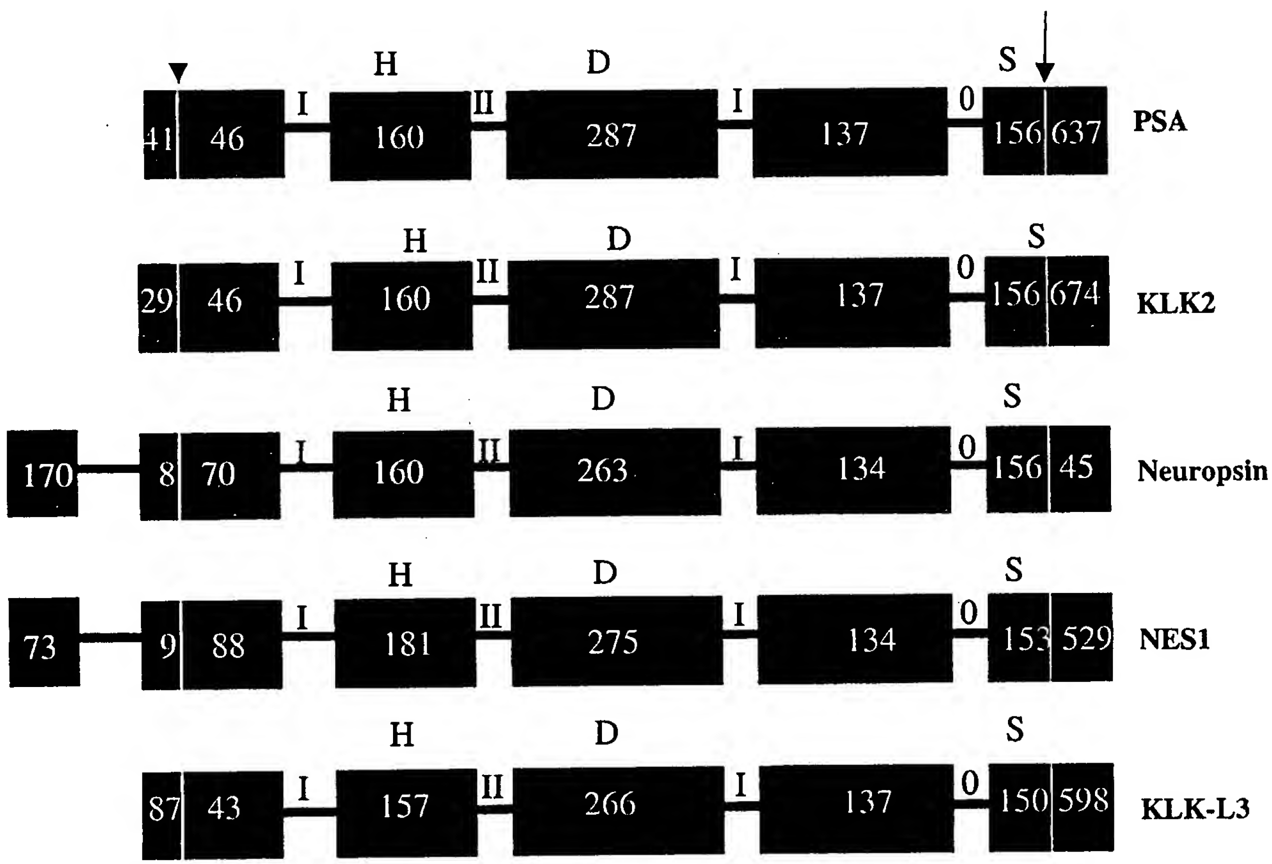

H
D
S
I
II
0
41
46
160
287
137
156
637
PSA
29
674
KLK2
170
8
70
263
134
45
Neuropsin
73
9
88
181
275
153
529
NES1
87
43
157
266
150
598
KLK-L3

FIGURE 22

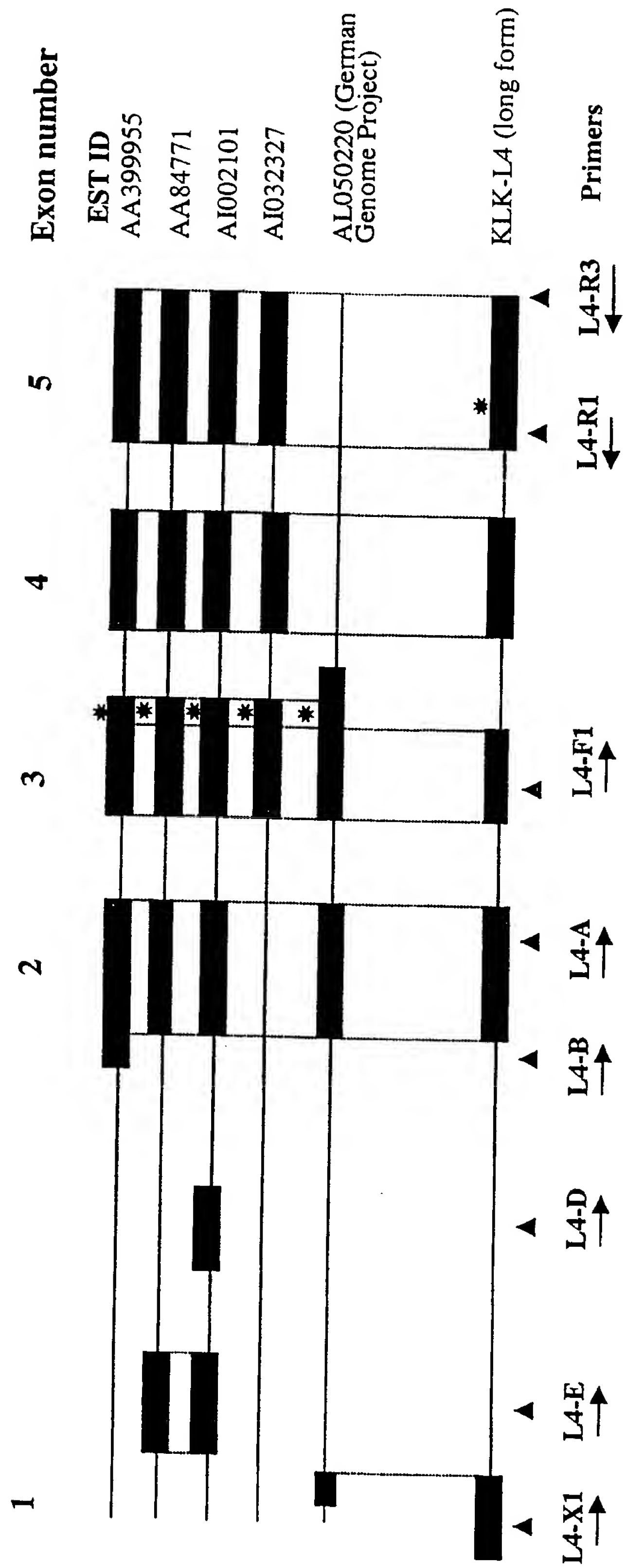

Exon number
1
2
3
4
5
EST ID
AA399955
AA84771
AI002101
AI032327
AL050220 (German Genome Project)
KLK-L4 (long form)
Primers
L4-X1
L4-E
L4-D
L4-B
L4-A
L4-F1
L4-R1
L4-R3

FIGURE 23

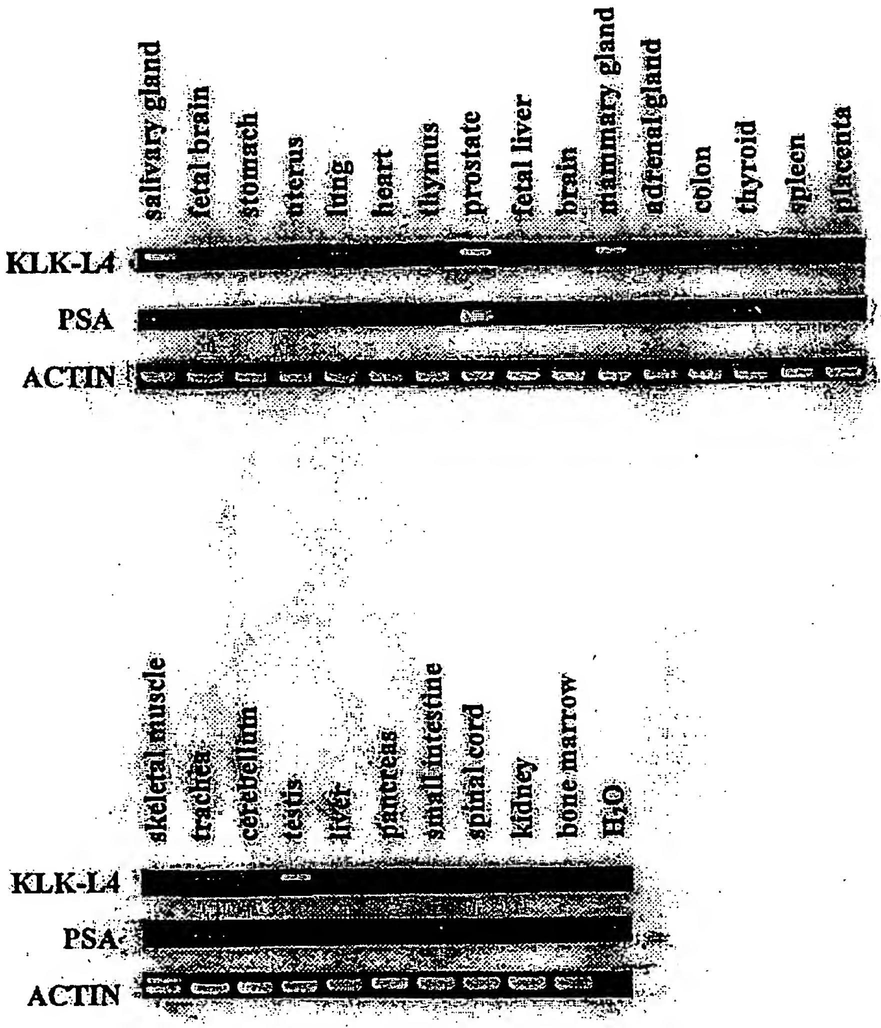

salivary gland
fetal brain
stomach
uterus
lung
heart
thymus
prostate
fetal liver
brain
mammary gland
adrenal gland
colon
thyroid
spleen
placenta
KLK-L4
PSA
ACTIN
skeletal muscle
trachea
cerebellum
testis
liver
pancreas
small intestine
spinal cord
kidney
bone marrow
H_2O
KLK-L4
PSA
ACTIN

FIGURE 24

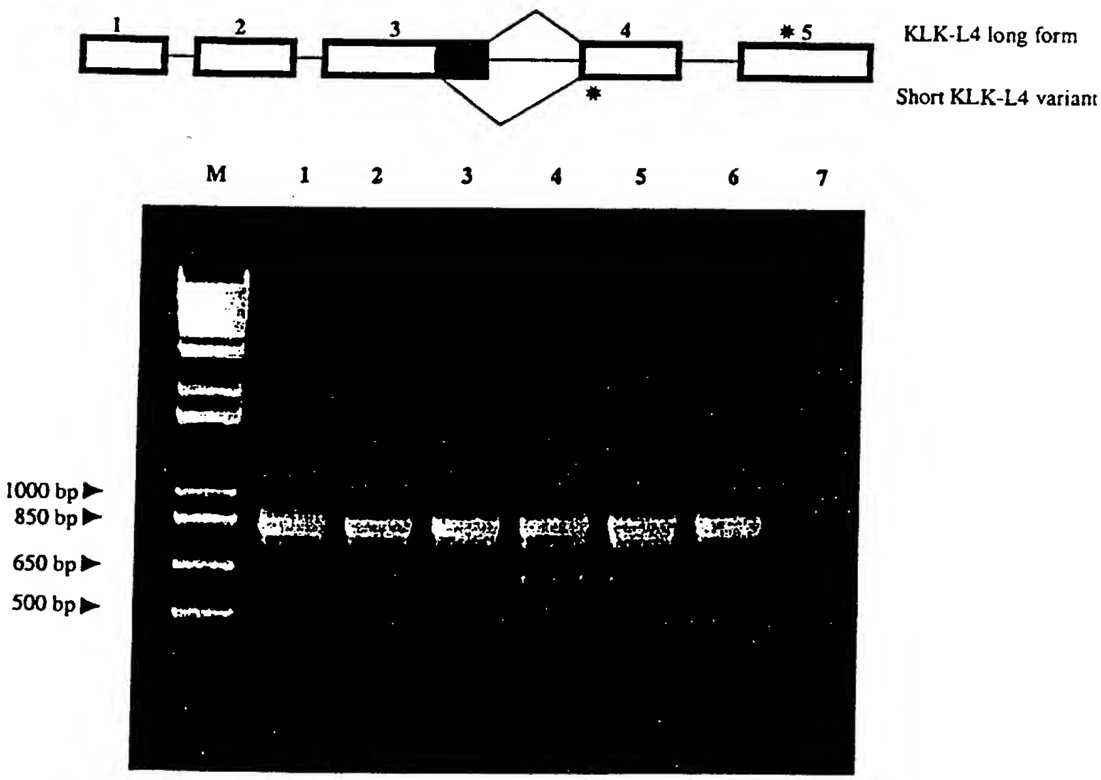

1
2
3
4
✱ 5
KLK-L4 long form
✱
Short KLK-L4 variant
M 1 2 3 4 5 6 7
1000 bp
850 bp
650 bp
500 bp

FIGURE 25

TCAGGCCCCGCCCGCCCTGCCCTCCCCTCCCGATCCCGGAGCC ATG TGG CCC CTG GCC
M W P L A
CTA GTG ATC GCC TCC CTG ACC TTG GCC TTG TCA GGA G...gtaaga.... intron 1 ..... ttaccag
L V I A S L T L A L S G

GT GTC TCC CAG GAG TCT TCC AAG GTT CTC AAC ACC AAT GGG ACC AGT GGG TTT
G V S Q E S S K V L N T N G T S G F

CTC CCA GGT GGC TAC ACC TGC TTC CCC CAC TCT CAG CCC TGG CAG GCT GCC
L P G G Y T C F P H S Q P W Q A A

CTA CTA GTG CAA GGG CGG CTA CTC TGT GGG GGA GTC CTG GTC CAC CCC AAA
L L V Q G R L L C G G V L V H P K

TGG GTC CTC ACT GCC GCA CAC TGT CTA AAG GA gtatgt ..... intron 2....... cacag G GGG
W V L T A A $\boxed{H}$ C L K E G

CTC AAA GTT TAC CTA GGC AAG CAC GCC CTA GGG CGT GTG GAA GCT GGT GAG
L K V Y L G K H A L G R V E A G E

CAG GTG AGG GAA GTT GTC CAC TCT ATC CCC CAC CCT GAA TAC CGG AGA AGC
Q V R E V V H S I P H P E Y R R S

CCC ACC CAC CTG AAC CAC GAC CAT GAC ATC ATG CTT CTG GAG CTG CAG TCC
P T H L N H D H $\boxed{D}$ I M L L E L Q S

CCG GTC CAG CTC ACA GGC TAC ATC CAA ACC CTG CCC CTT TCC CAC AAC AAC CGC
P V Q L T G Y I Q T L P L S H N N R

CTA ACC CCT GGC ACC ACC TGT CGG GTG TCT GGC TGG GGC ACC ACC ACC AGC
L T P G T T C R V S G W G T T T S

CCC CAG G gtatgcac... intron 3..... tcccc ag TG AAT TAC CCC AAA ACT CTA CAA TGT GCC
P Q V N Y P K T L Q C A

AAC ATC CAA CTT CGC TCA GAT GAG GAG TGT CGT CAA GTC TAC CCA GGA AAG
N I Q L R S D E E C R Q V Y P G K

ATC ACT GAC AAC ATG TTG TGT GCC GGC ACA AAA GAG GGT GGC AAA GAC TCC
I T D N M L C A G T K E G G K D S

TGT GAG gtatgca... intron 4..... aactcag GGT GAC TCT GGG GGC CCC CTG GTC TGT AAC
C E G D $\boxed{S}$ G G P L V C N

AGA ACA CTG TAT GGC ATC GTC TCC TGG GGA GAC TTC CCA TGT GGG CAA CCT
R T L Y G I V S W G D F P C G Q P
GAC CGG CCT GGT GTC TAC ACC CGT GTC TCA AGA TAC GTC CTG TGG ATC CGT
D R P G V Y T R V S R Y V L W I R

FIGURE 25 (CONT'D)

GAA ACA ATC CGA AAA TAT GAA ACC CAG CAG CAA AAA TGG TTG AAG GGC CCA
E T I R K Y E T Q Q Q K W L K G P

CAA TAA AAGTTGAGAAATGTACCGGCTTCCATCCTGTCACCATGACTTCCTCAC
Q

ATGGTCTGCTTAGCCCTTCTCTGCTCCTTATTCCCAGTGTTCCATTTGAACCAGTGATCCATGTC
CTGAAAAATGCTCAATCTCAGCTAACATTCCATGTTTCAGAAGCATTCAGGCACTGCCAGGCT
TGCAGTCTCCCAGATGTTGCATCCCTGAAACATCTCAACAACCTGAATGTCCCAACCCAGACA
ATGGCCCAGGTCTCTCAACTTCATCAGTGTGGCTTCTATGAGCCCAGATCACCACCTGAACGT
TCTGTCTGTGGCACATTCTTAAATATTTCCATCAGCCCATCTCAACAATATATGTCC<u>TATAAA</u>T
GGACCATCCTTGACA

FIGURE 26

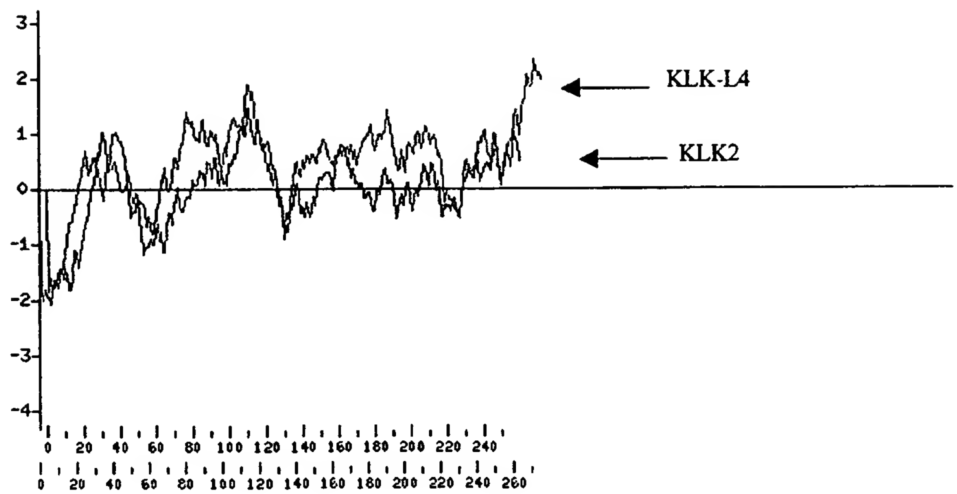

KLK-L4
KLK2
3
2
1
0
-1
-2
-3
-4
0 20 40 60 80 100 120 140 160 180 200 220 240
0 20 40 60 80 100 120 140 160 180 200 220 240 260

FIGURE 27

```
                          1             15 16            30 31            45 46            60 61            75 76            90
 1 KLK-L1/prostase  ----MATAGN PWGWFLG----YLIL GVAG----------- ----SLVSG------ -----------SCSQ IINGEDCSPHSQPWQ  45
 2 EMSP             ----MATAGN PWGWFLG----YLIL GVAG----------- ----SLVSG------ -----------SCSQ IINGEDCSPHSQPWQ  45
 3 KLK-L2           ----MATARP PWMWVLCALITALLL GVTEHVLANNDVSCD HPSNTVPSGSNQDLG AGAGEDARSDDSSSR IINGSDCDMHTQPWQ  81
 4 PSA              ---------- MWVPVVF---LTLSV TWIG----------- ----AAPLI------ ---------L---SR IVGGWECEKHSQPWQ  39
 5 KLK2             ---------- MWDLVLS---IALSV GCTG----------- ----AVPLI------ ---------Q---SR IVGGWECEKHSQPWQ  39
 6 KLK1             ---------- MWFLVLC---LALSL GGTG----------- ----AAPPI------ ---------Q---SR IVGGWECEQHSQPWQ  39
 7 trypsinogen      ---------- -MNPLLI---LTFVA AALA----------- ----APFDD------ ---------D---DK IVGGYNCEENSVPYQ  38
 8 zyme/protease M  ---------- -MKKLMV----VLSL IAAA----------- ---WAEEQNK----- --------------- LVHGGPCDKTSHPYQ  36
 9 KLK-L4           --------MW PLALVIA----SLTL ALSG----------- ---GVSQESSKV--- ------LNTNGTSGF LPGGYTCFPHSQPWQ  50
10 TLSP             --------MR ILQLILLALATGLVG GETR----------- ----I---------- --------------- -IKGFECKPHSQPWQ  36
11 neuropsin        --MGRPRPRA AKTWMFLLLLGGAWA GHSR----------- ----AQEDK------ --------------- VLGGHECQPHSQPWQ  47
12 NES1       MRAPHLHLSAASGAR ALAKLLPLLMAQLWA AEAA----------- ----LLPQN------ ---------DTRLDP EAYGAPCARGSQPWQ  60

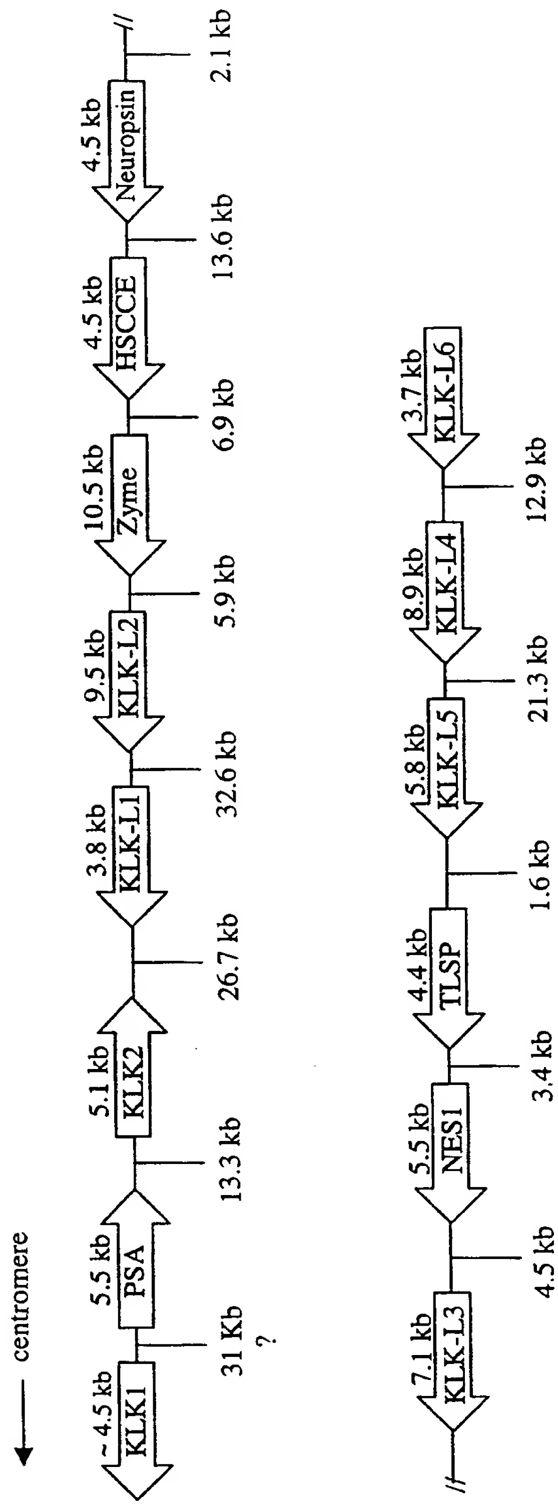
                  91            105 106           120 121           135 136           150 151           165 166           180
 1 prostase        AALVM-ENELFCSGV LVHPQWVLSAAHCFQ NSYTIGLGLHSLEAD QEPGSQMVEASLSVR HPEYNRPLLAN---- -------DLMLIKLD 123
 2 EMSP             AALVM-ENELFCSGV LVHPQWVLSAAHCFQ NSYTIGLGLHSLEAD QEPGSQMVEASLSVR HPEYNRPLLAN---- -------DLMLIKLD 123
 3 KLK-L2           AALLLRPNQLYCGAV LVHPQWLLTAAHCRK KVFRVRLGHYSLSPV YESGQQMFQGVKSIP HPGYSHPGHSN---- -------DLMLIKLN 160
 4 PSA              VLVAS-RGRAVCGGV LVHPQWVLTAAHCIR NKSVILLGRHSLFHP -EDTGQVFQVSHSFP HPLYDMSLLKNRFLR PGDDSSHDLMLLRLS 127
 5 KLK2             VAVYS-HGWAHCGGV LVHPQWVLTAAHCLK KNSQVWLGRHNLFEP -EDTGQRVPVSHSFP HPLYNMSLLKHQSLR PDEDSSHDLMLLRLS 127
 6 KLK1             AALYH-FSTFQCGGI LVHRQWVLTAAHCIS DNYQLWLGRHNLFDD -ENTAQFVHVSESFP HPGFNMSLLENHTRQ ADEDYSHDLMLLRLT 127
 7 trypsinogen      VSLNS--GYHFCGGS LINEQWVVSAGHCYK SRIQVRLGEHNIEVL -EGNEQFINAAKIIR HPQYDRKTLNN---- -------DIMLIKLS 114
 8 zyme             AALYT-SGHLLCGGV LIHPLWVLTAAHCKK PNLQVFLGKHNLRQR -ESSQEQSSVVRAVI HPDYDAA-------- ---SHDQDIMLLRLA 113
 9 KLK-L4           AALLV-QGRLLCGGV LVHPKWVLTAAHCLK EGLKVYLGKHALGRV -EAGEQVREVVHSIP HPEYRRSPTHL---- ---NHDHDIMLLELQ 131
10 TLSP             AALFE-KTRLLCGAT LIAPRWLLTAAHCLK PRYIVHLGQHNLQKE -EGCEQTRTATESFP HPGFNNSLPNK---- ---DHRNDIMLVKMA 117
11 neuropsin        AALFQ-GQQLLCGGV LVGGNWVLTAAHCKK PKYTVRLGDHSLQNK -DGPEQEIPVVQSIP HPCYNSSDVE----- ---DHNHDLMLLQLR 127
12 NES1             VSLFN-GLSFHCAGV LVDQSWVLTAAHCGN KPLWARVGDDHLLLL Q--GEQLRRTTRSVV HPKYHQGS-GPILPR R--TDEHDLMLLKLA 144

                  181           195 196           210 211           225 226           240 241           255 256           270
 1 prostase        ESVS-ESDTIRSISI ASQCPTAG-NSCLVS GWGLLANG--RMPTV LQCVNVSVVSEEVCS KLYDPLYHPSMFCAG GGHDQKDSCNGDSGG 209
 2 EMSP             ESVS-ESDTIRSISI ASQCPTAG-NSCLVS GWGLLANG--RMPTV LQCVNVSVVSEEVCS KLYDPLYHPSMFCAG GGHDQKDSCNGDSGG 209
 3 KLK-L2           RRIR-PTKDVRPINV SSHCPSAG-TKCLVS GWGTTKSPQVHFPKV LQCLNISVLSQKRCE DAYPRQIDDTMFCAG DKAG-RDSCQGDSGG 247
 4 PSA              EPAE-LTDAVKVMDL PTQEPALG-TTCYAS GWGSIEPEEFLTPKK LQCVDLHVISNDVCA QVHPQKVTKFMLCAG RWTGGKSTCSGDSGG 215
 5 KLK2             EPAK-ITDVVKVLGL PTQEPALG-TTCYAS GWGSIEPEEFLRPRS LQCVSLHLLSNDMCA RAYSEKVTEFMLCAG LWTGGKDTCGGDSGG 215
 6 KLK1             EPADTITDAVKVVEL PTEEPEVG-STCLAS GWGSIEPENFSFPDD LQCVDLKILPNDECK KAHVQKVTDFMLCVG HLEGGKDTCVGDSGG 216
 7 trypsinogen      SRAV-INARVSTISL PTAPPATG-TKCLIS GWGNTASSGADYPDE LQCLDAPVLSQAKCE ASYPGKITSNMFCVG FLEGGKDSCQGDSGG 202
 8 zyme             RPAK-LSELIQPLPL ERDCSANT-TSCHIL GWGKTADG--DFPDT IQCAYIHLVSREECE HAYPGQITQNMLCAG DEKYGKDSCQGDSGG 199
 9 KLK-L4           SPVQ-LTGYIQTLPL SHNNRLTPGTTCRVS GWGTTTSPQVNYPKT LQCANIQLRSDEECR QVYPGKITDNMLCAG TKEGGKDSCEGDSGG 220
10 TLSP             SPVS-ITWAVRPLTL SSRCVTAG-TSCLIS GWGSTSSPQLRLPHT LRCANITIIEHQKCE NAYPGNITDTMVCAS VQEGGKDSCQGDSGG 205
11 neuropsin        DQAS-LGSKVKPISL ADHCTQPG-QKCTVS GWGTVTSPRENFPDT LNCAEVKIFPQKKCE DAYPGQITDGMVCAG SSKG-ADTCQGDSGG 214
12 NES1             RPVVP-GPRVRALQL PYRCAQPG-DQCQVA GWGTTAARRVKYNKG LTCSSITILSPKECE VFYPGVVTNNMICAG LDRG-QDPCQSDSGG 231
```

FIGURE 27 (CONT'D)

```
                271           285 286           300 301           315 316           330 331
 1 prostase     PLICNGYLQGLVSFG KAPCGQVGVPGVYTN LCKFTEWIEKTVQAS ------------    254
 2 EMSP         PLICNGYLQGLVSFG KAPCGQVGVPGVYTN LCKFTEWIEKTVQAS ------------    254
 3 KLK-L2       RVVCNGSLQGLVSWG DYPCARPNRPGVYTN LCKFTKWIQETIQAN S-----------    293
 4 PSA          PLVCNGVLQGITSWG SEPCALPERPSLYTK VVHYRKWIKDTIVAN P-----------    261
 5 KLK2         PLVCNGVLQGITSWG PEPCALPEKPAVYTK VVHYRKWIKDTIAAN P-----------    261
 6 KLK1         PLMCDGVLQGVTSWG YVPCGTPNKPSVAVR VLSYVKWIEDTIAEN S-----------    262
 7 trypsinogen  RVVCNGQLQGVVSWG -DGCAQKNKPGVYTK VYNYVKWIKNTIAAN S-----------    247
 8 zyme         PLVCGDHLRGLVSWG NIPCGSKEKPGVYTN VCRYTNWIQKTIQAK ------------    244
 9 KLK-L4       PLVCNRTLYGIVSWG DFPCGQPDRPGVYTR VSRYVLWIRETIRKY ETQQQKWLKGPQ    277
10 TLSP         PLQCNQSLQGIISWG QDPCAITRKPGVYTK VCKYVDWIQETMKNN ------------    250
11 neuropsin    PLVCDGALQGITSWG SDPCGRSDKPGVYTN ICRYLDWIKKIIGSK G-----------    260
12 NES1         PLVCDETLQGILSWG VYPCGSAQHPAVYTQ ICKYMSWINKVIRSN ------------    276
                .  ♦    .       .
                                ♦
```

FIGURE 28



centromere
KLK1
~4.5 kb
31 Kb
?
PSA
5.5 kb
13.3 kb
KLK2
5.1 kb
26.7 kb
KLK-L1
3.8 kb
32.6 kb
KLK-L2
9.5 kb
5.9 kb
Zyme
10.5 kb
6.9 kb
HSCCE
4.5 kb
13.6 kb
Neuropsin
4.5 kb
2.1 kb
KLK-L3
7.1 kb
4.5 kb
NES1
5.5 kb
3.4 kb
TLSP
4.4 kb
1.6 kb
KLK-L5
5.8 kb
21.3 kb
KLK-L4
8.9 kb
12.9 kb
KLK-L6
3.7 kb

FIGURE 29

```
             +------TLSP
        +----4
        !    +--------------KLK-L3
     +-13
     !  !  +-------------neuropsin
     !  +-12
  +-14     +----------------------NES1
  !  !
  !  !  +------------zyme
  !  +-11
  !     +-------------KLK-L4
  !
  !                 +-----PSA
  !             +---2
  !  +----------3   +---KLK2
  !  !          !
  !  !          +--------KLK1
-15-10
  !  !  +------------trypsinogen I
  !  !  !
  !  !  !         +----------------complement factor D
  !  +--9      +--6
  !     !  +---7  +---------------------granzyme A
  !     !  !   !
  !     +--8   +---------------------cathepsin G
  !        !
  !        +---------------------chymotrypsin
  !
  !                  +prostase/KLK-L1
  !    +-------------1
  +----5             +EMSP
       !
       +----------KLK-L2
```

FIGURE 30

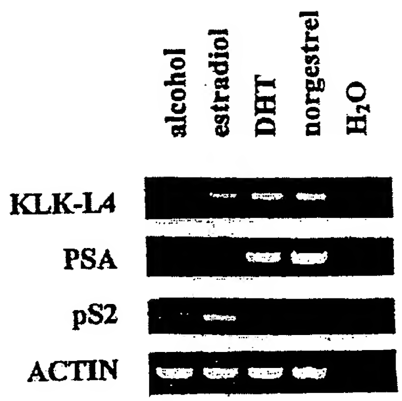


FIGURE 31

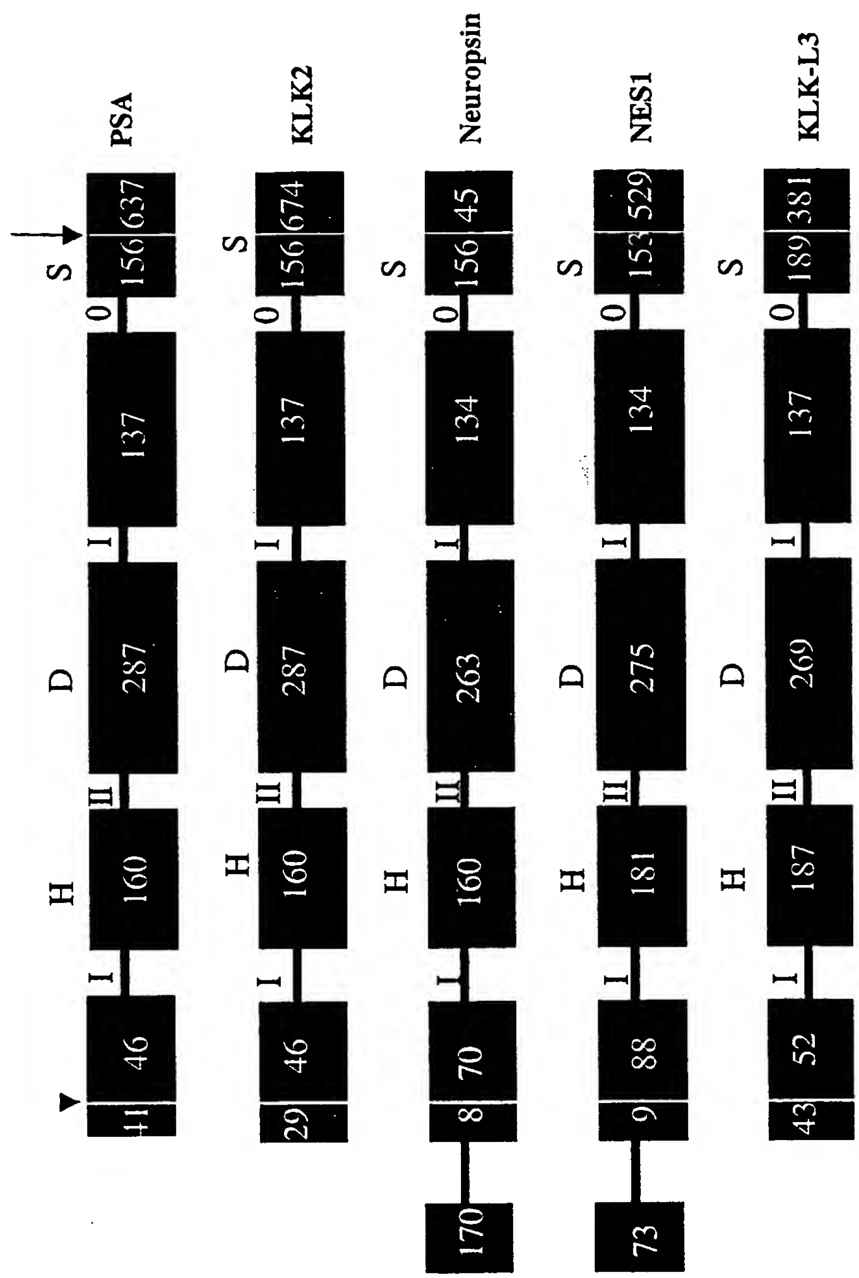

PSA
41 46 160 287 137 156 637
KLK2
29 46 160 287 137 156 674
Neuropsin
170 8 70 160 263 134 156 45
NES1
73 9 88 181 275 134 153 529
KLK-L3
43 52 187 269 137 189 381
I
II
I
0
H
D
S

FIGURE 32

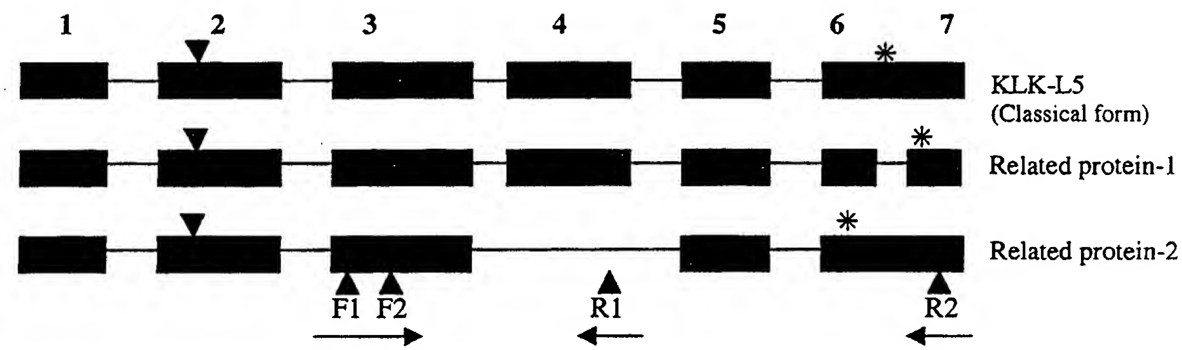

1
2
3
4
5
6
7
KLK-L5
(Classical form)
Related protein-1
Related protein-2
F1
F2
R1
R2

FIGURE 33

GCAGGTAGGTGGACGGAGAGATAGCAGCGACGAGGACAGGCCAAACAGTGACAGCCACG
TAGAGGATCTGGCAGACAAAGAGACAAGGTGAGAAGGAG gtagg.........Intron 1......
....tgacactcccccag ACTTTGGAAGTGACCCACC ATG
M
GGG CTC AGC ATC TTT TTG CTC CTG TGT GTT CTT G gtgagttctcccg
G L S I F L L L C V L
gagcagggagagggca........... Intron 2 ....cctgtctgtctccag GG CTC
G L
AGC CAG GCA GCC ACA CCG AAG ATT TTC AAT GGC ACT GAG TGT GGG
S Q A A T P K I F N G T E C G
CGT AAC TCA CAG CCG TGG CAG GTG GGG CTG TTT GAG GGC ACC AGC
R N S Q P W Q V G L F E G T S
CTG CGC TGC GGG GGT GTC CTT ATT GAC CAC AGG TGG GTC CTC ACA
L R C G G V L I D H R W V L T
GCG GCT CAC TGC AGC GGC AG gtaagtcccttcc...........intron3..................
A A H C S G S
.ccgtcgccaccggcag C AGG TAC TGG GTG CGC CTG GGG GAA CAC AGC
R Y W V R L G E H S
CTC AGC CAG CTC GAC TGG ACC GAG CAG ATC CGG CAC AGC GGC TTC
L S Q L D W T E Q I R H S G F
TCT GTG ACC CAT CCC GGC TAC CTG GGA GCC TCG ACG AGC CAC GAG
S V T H P G Y L G A S T S H E
CAC GAC CTC CGG CTG CTG CGG CTG CGC CTG CCC GTC CGC GTA ACC
H D L R L L R L R L P V R V T
AGC AGC GTT CAA CCC CTG CCC CTG CCC AAT GAC TGT GCA ACC GCT
S S V Q P L P L P N D C A T A
GGC ACC GAG TGC CAC GTC TCA GGC TGG GGC ATC ACC AAC CAC CCA
G T E C H V S G W G I T N H P
CGG A gtaaggggcccagggccaggg...............................intron 4 ....
R
.gaccctgcagcacgcatgttctctctccag AC CCA TTC CCG GAT CTG CTC
N P F P D L L
CAG TGC CTC AAC CTC TCC ATC GTC TCC CAT GCC ACC TGC CAT GGT
Q C L N L S I V S H A T C H G
GTG TAT CCC GGG AGA ATC ACG AGC AAC ATG GTG TGT GCA GGC GGC
V Y P G R I T S N M V C A G G
GTC CCG GGG CAG GAT GCC TGC CAG gtgagcc........... Intron 5 ....
V P G Q D A C Q
.aaaacagaaataagatgtctcccttgttcagacagtacttctcttcccttccag GGT
G
GAT TCT GGG GGC CCC CTG GTG TGT GGG GGA GTC CTT CAA GGT CTG
D S G G P L V C G G V L Q G L
GTG TCC TGG GGG TCT GTG GGG CCC TGT GGA CAA GAT GGC ATC CCT
V S W G S V G P C G Q D G I P
GGA GTC TAC ACC TAT ATT TGC AA(G TAT GTG GAC TGG ATC CGG ATG
G V Y T Y I C K Y V D W I R M
ATC ATG AGG AAC AAC TGA CCTGTTTCCTCCACCTCCACCCCCACCCCTTAACTT
I M R N N
GGGTACCCCTCTGGCCCTCAGAGCACCAATATCTCCTCCATCACTTCCCCTAG) CTCCAC
TCTTGTTGGCCTGGGAACTTCTTGGAACTTTAACTCCTGCCAGCCCTTC TAA GACCCACG
AGCGGGGTGAGAGAAGTGTGCAATAGTCTGGAATAAATATAAATGAAGGAGGGGC

FIGURE 34

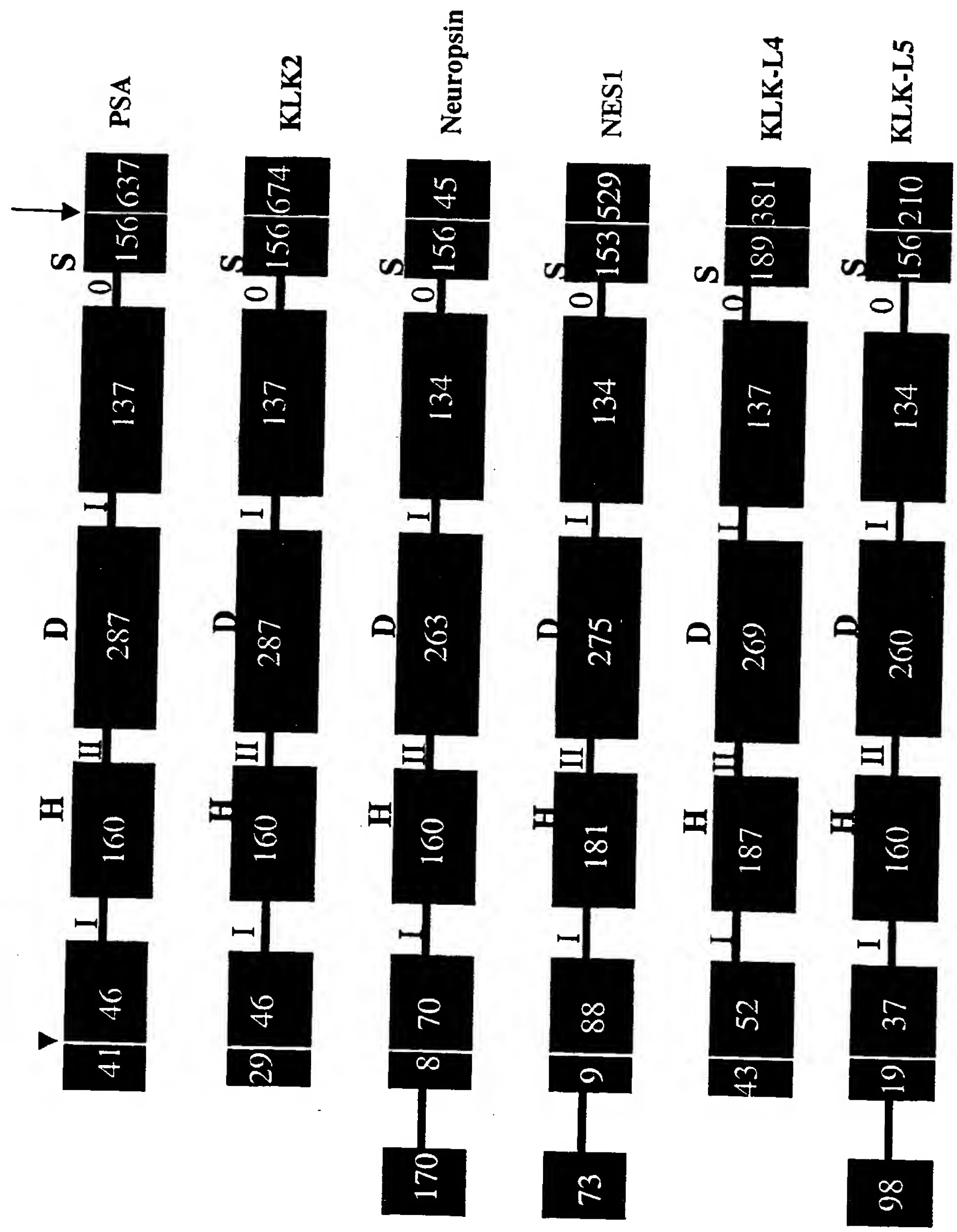


FIGURE 35

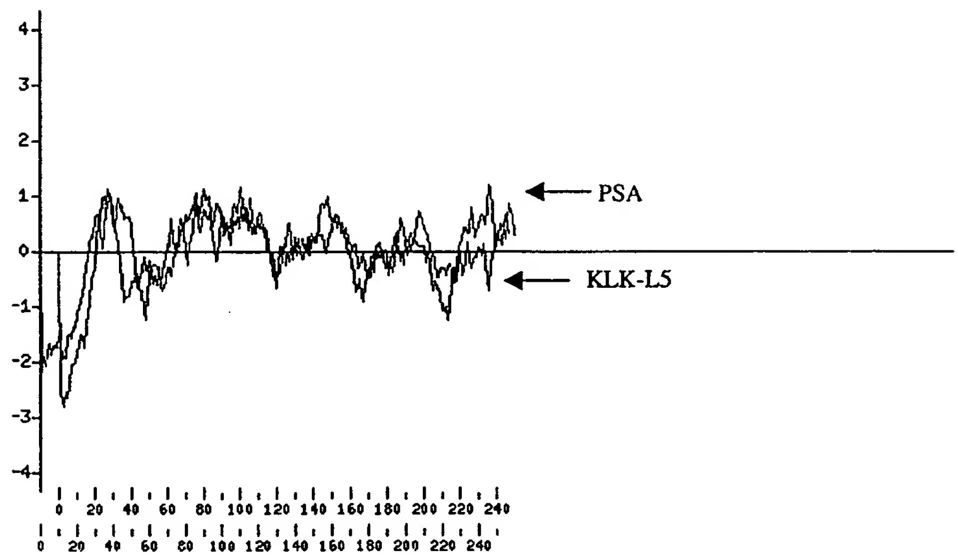

4
3
2
1
0
-1
-2
-3
-4
PSA
KLK-L5
0 20 40 60 80 100 120 140 160 180 200 220 240
0 20 40 60 80 100 120 140 160 180 200 220 240

Figure 36

```
              1           15 16          30 31          45 46          60 61          75 76          90
 1 PSA        --------------- MWVPVV-FLTLSVTW IGAAPLILSR----- -----IVGGWECEKH SQPWQVLVASRGRAV CGGVLVHPQWVLTAA    64
 2 hK2        --------------- MWDLVL-SIALSVGC TGAVPLIQSR----- -----IVGGWECEKH SQPWQVAVYSHGWAH CGGVLVHPQWVLTAA    64
 3 hK1        --------------- MWFLVL-CLALSLGG TGAAPPIQSR----- -----IVGGWECEQH SQPWQAALYHFSTFQ CGGILVHRQWVLTAA    64
 4 prostase   ---------MATAGN PWGWFLGYLILGVAG S-LVSGSCSQ----- -----IINGEDCSPH SQPWQAALVMENELF CSGVLVHPQWVLSAA    70
 5 zyme       --------------- MKKLMV---VLSLIA A-AWAEEQNK----- -----LVHGGPCDKT SHPYQAALYTSGHLL CGGVLIHPLWVLTAA    61
 6 TLSP       --------------- MRILQLILLALATGL VGGETR--------- -----IIKGFECKPH SQPWQAALFEKTRLL CGATLIAPRWLLTAA    61
 7 KLK-L4     --------------- MWPLALVIASLTLAL SGGVSQESSKVLNTN GTSGFLPGGYTCFPH SQPWQAALLVQGRLL CGGVLVHPKWVLTAA    75
 8 NES1       MRAPHLHLSAASGAR ALAKLLPLLMAQLWA AEAALLPQNDT---- -RLDPEAYGAPCARG SQPWQVSLFNGLSFH CAGVLVDQSWVLTAA    85
 9 KLK-L5     --------------- -MGLSIFLLLCVLGL SQAATPKIFN----- --------GTECGRN SQPWQVGLFEGTSLR CGGVLIDHRWVLTAA    61
10 neuropsin  ----------MGRPR PRAAKTWMFLLLLGG AWAGHSRAQE----- ---DKVLGGHECQPH SQPWQAALFQGQQLL CGGVLVGGNWVLTAA    72

              91         105 106        120 121         135 136         150 151         165 166         180
 1 PSA        HCIRNKSVILLGRHS LFHPEDTG-QVFQVS HSFPHPLYDMSLLKN RFLRPGDDSSHDLML LRLSEPAE-LTDAVK VMDLPTQ-EPALGTT   151
 2 hK2        HCLKKNSQVWLGRHN LFEPEDTG-QRVPVS HSFPHPLYNMSLLKH QSLRPDEDSSHDLML LRLSEPAK-ITDVVK VLGLPTQ-EPALGTT   151
 3 hK1        HCISDNYQLWLGRHN LFDDENTA-QFVHVS ESFPHPGFNMSLLEN HTRQADEDYSHDLML LRLTEPADTITDAVK VVELPTE-EPEVGST   152
 4 prostase   HCFQNSYTIGLGLHS LEADQEPGSQMVEAS LSVRHPEYNRPLLAN -----------DLML IKLDESVS-ESDTIR SISIASQ-CPTAGNS   147
 5 zyme       HCKKPNLQVFLGKHN LRQRESSQ-EQSSVV RAVIHPDYDAASHDQ -----------DIML LRLARPAK-LSELIQ PLPLERD-CSANTTS   137
 6 TLSP       HCLKPRYIVHLGQHN LQ-KEEGCEQTRTAT ESFPHPGFNNSLPNK -------DHRNDIML VKMASPVS-ITWAVR PLTLSSR-CVTAGTS   141
 7 KLK-L4     HCLKEGLKVYLGKHA LG-RVEAGEQVREVV HSIPHPEYRRSPTHL -------NHDHDIML LELQSPVQ-LTGYIQ TLPLSHNNRLTPGTT   156
 8 NES1       HCGNKPLWARVGDDH LL-LLQG-EQLRRTT RSVVHPKYHQGSGPI L---PRRTDEHDLML LKLARPVV-PGPRVR ALQLPYR-CAQPGDQ   168
 9 KLK-L5     HCSGSRYWVRLGEHS LS-QLDWTEQIRHSG FSVTHPGYLGAS--- ------TSHEHDLRL LRLRLPVR-VTSSVQ PLPLPND-CATAGTE   139
10 neuropsin  HCKKPKYTVRLGDHS LQ-NKDGPEQEIPVV QSIPHPCYNSSD--- -----VEDHNHDLML LQLRDQAS-LGSKVK PISLADH-CTQPGQK   151

              181         195 196         210 211         225 226         240 241         255 256         270
 1 PSA        CYASGWGSIEPEEFL TPKKLQCVDLHVISN DVCAQVHPQKVTKFM LCAGRWTGGKSTCSG DSGGPLVCNGVLQGI TSWG-SEPCALPERP   240
 2 hK2        CYASGWGSIEPEEFL RPRSLQCVSLHLLSN DMCARAYSEKVTEFM LCAGLWTGGKDTCGG DSGGPLVCNGVLQGI TSWG-PEPCALPEKP   240
 3 hK1        CLASGWGSIEPENFS FPDDLQCVDLKILPN DECKKAHVQKVTDFM LCVGHLEGGKDTCVG DSGGPLMCDGVLQGV TSWG-YVPCGTPNKP   241
 4 prostase   CLVSGWGLLA--NGR MPTVLQCVNVSVVSE EVCSKLYDPLYHPSM FCAGGGHDQKDSCNG DSGGPLICNGYLQGL VSFG-KAPCGQVGVP   234
 5 zyme       CHILGWGKTA--DGD FPDTIQCAYIHLVSR EECEHAYPGQITQNM LCAGDEKYGKDSCQG DSGGPLVCGDHLRGL VSWG-NIPCGSKEKP   224
 6 TLSP       CLISGWGSTSSPQLR LPHTLRCANITIIEH QKCENAYPGNITDTM VCASVQEGGKDSCQG DSGGPLVCNQSLQGI ISWG-QDPCAITRKP   230
 7 KLK-L4     CRVSGWGTTTSPQVN YPKTLQCANIQLRSD EECRQVYPGKITDNM LCAGTKEGGKDSCEG DSGGPLVCNRTLYGI VSWG-DFPCGQPDRP   245
 8 NES1       CQVAGWGTTAARRVK YNKGLTCSSITILSP KECEVFYPGVVTNNM ICAGLDR-GQDPCQS DSGGPLVCDETLQGI LSWG-VYPCGSAQHP   256
 9 KLK-L5     CHVSGWGITNHPRNP FPDLLQCLNLSIVSH ATCHGVYPGRITSNM VCAGGVP-GQDACQG DSGGPLVCGGVLQGL VSWGSVGPCGQDGIP   228
10 neuropsin  CTVSGWGTVTSPREN FPDTLNCAEVKIFPQ KKCEDAYPGQITDGM VCAGSSK-GADTCQG DSGGPLVCDGALQGI TSWG-SDPCGRSDKP   239
                                                                            ↑
```

Figure 36 cont'd

```
                271            285 286           300 301
 1 PSA          SLYTKVVHYRKWIKD TIVANP--------- --      261
 2 hK2          AVYTKVVHYRKWIKD TIAANP--------- --      261
 3 hK1          SVAVRVLSYVKWIED TIAENS--------- --      262
 4 prostase     GVYTNLCKFTEWIEK TVQAS---------- --      254
 5 zyme         GVYTNVCRYTNWIQK TIQAK---------- --      244
 6 TLSP         GVYTKVCKYVDWIQE TMKNN---------- --      250
 7 KLK-L4       GVYTRVSRYVLWIRE TIRKYETQQQKWLKG PQ      277
 8 NES1         AVYTQICKYMSWINK VIRSN---------- --      276
 9 KLK-L5       GVYTYICKYVDWIRM IMRNN---------- --      248
10 neuropsin    GVYTNICRYLDWIKK IIGSKG--------- --      260
                      ♦
```

FIGURE 37

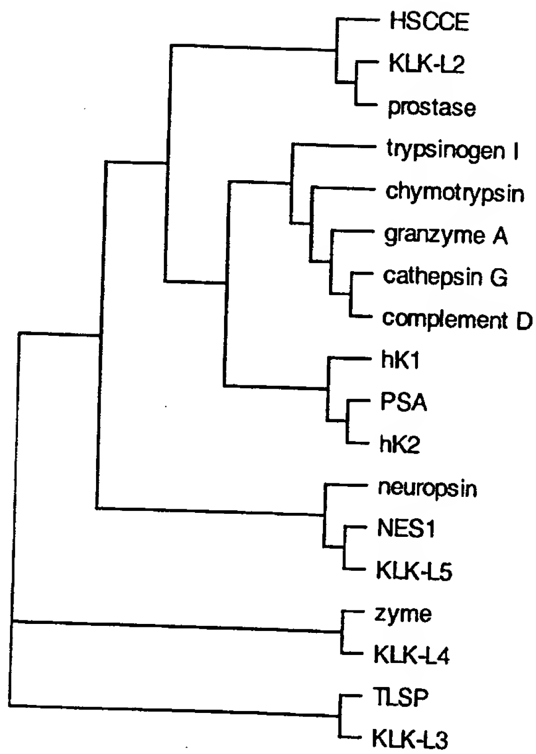

HSCCE
KLK-L2
prostase
trypsinogen I
chymotrypsin
granzyme A
cathepsin G
complement D
hK1
PSA
hK2
neuropsin
NES1
KLK-L5
zyme
KLK-L4
TLSP
KLK-L3

FIGURE 38

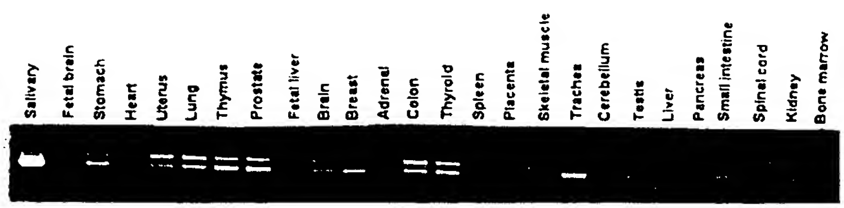

Salivary
Fetal brain
Stomach
Heart
Uterus
Lung
Thymus
Prostate
Fetal liver
Brain
Breast
Adrenal
Colon
Thyroid
Spleen
Placenta
Skeletal muscle
Trachea
Cerebellum
Testis
Liver
Pancreas
Small intestine
Spinal cord
Kidney
Bone marrow

FIGURE 39

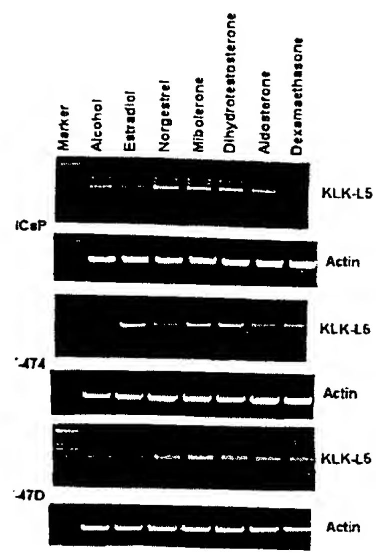

Marker
Alcohol
Estradiol
Norgestrel
Mibolerone
Dihydrotestosterone
Aldosterone
Dexamethasone
KLK-L5
Actin
KLK-L6
Actin
KLK-L6
Actin
ICaP
'474
'47D

FIGURE 40

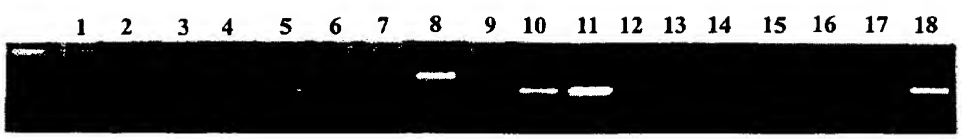


FIGURE 41

ATCGTGTAAT CACCGCCACA TCCAGTGCAA AGCTGATTCG TCACCACAGA GCAGCTCCCT
CCTGCCACCC CATCCCTGGG TCCCAAGAGA ACCCTTTCTT AAAAGAGGGA GTTCTTGACG
GGTGTGGTGG CTCATGCCTG TAATCCTTGC ACTTTGGGAG GCCAAGGAGG GTGGATCATT
TGAGGTCAGG AGTTTGAGAC CAGACTGGCC AACATGGTGA AACCCTGTCT TTACTAAAAA
TACAAAAAAA TGAGCGGGGC ATGGTGGTGG GTGCCTATAG CCCCAGCTAC TCAGGAGGCT
GAGGCAGGAG AATCGCTTGA ACCCAGGAGG CAGAGGTTGC AGTGAGCCGA GATTGAGCCA
CTGCACTCCA GCCGGGGCTA AAGAGTGAGA CTCTGTCTCA AAAAAAAAAA AAAGAAAAAG
AAAAAAAGAA AAAAAAATAA AATAAATAAA TAAATAAAAT AAATTTAAAA ATTTAAAAAT
AAAGAGGGGG TTCTTGTGTT GATGCCGAGC CTGAACCAAG GCAGAGGAGG CCGGGAAGGC
TTCCCAAGGC CTTCAGCTCA AAGCAGGGAG GCCCATAGTT AAACAGAAAC AGTTCAGGAA
TCACAGAAAG GCACCTGGGG AGAGATGGGT GTGTGGCTCC AGATGCAGGT GCCCAGACAG
TGCGTCCCCA GGTGTACAGA CAGACCCAGG CCAAGCTCCA GCTCAAAGAG CCAGCCTAGG
GGGGTGCCGA GGTGGAGGGA GGCTGAGTCA GGCTGAGGCC GGGGAACAGT TGGGGTAGCC
AAGGGAGGCA AGCAGCCTCC TGAGTCACCA CGTGGTCCAG GTACGGGGCT GCCCAGGCCC
AGAGACGGAC ACAAGCACTG GGGAATTTAA GGGGCTAGGG GAGGGGCTGA GGAGGGTAGG
CCCTCCCCCA AATGAGGATG GAACCCCCCC AACTCCAGAA CCCCCCTGCA GGCTGGCCAG
AATCCTTCCC CATCTCATTC ACTCTGTCTC TCCTGCTCTC TGCCGTCTCC TATTTTGAAT
TTCCAACCCC GTCTGTTAAG ACTGTCCTTC TGTCTCTGAA TCTCTGTCCC CTTCTCTTTC
TGGGTCTCTC TCCCTCTCCC TCTGGGTCTC TGTCCCCCTC TCTGGGTCTC TGTCACTCTC
TCTTTGCATC TCCAGCTCTC ACTTTGTCTC T**GCACCTAGC AGATCCCAAG CTGGGGAATG**
CCAGTTCTGG CACCAACCTT CCTGCTCCCT GCTGGGGCCT CTGCTCCCCC ATCTCTCAGG
AGTCGAAAGT GAGAAAGCAA GGTGGGCAGC TCTGCTCCAG GTCCAGGTAT CTCCCGCCCA
CCTCCTGCCC GTCCTCTATC CCACCCCTCC TCTCCATCTC TCCCTGGCGC TGCCATCTCT
CATCTAGGCC TCCGTCTCCT CTGTCATTGT CCCCATCCCC TGTAGGTGCC CATCCTTCCC
GTCTCCCCTC TGCCATCGGC CTGCCTGTCC CATCCTCTTT CTCCCACCAT GTCCCGTTCT
CTTCCACGTC TCATGCCCGC ACTGCCTTCA TCATCATCGC TGTTGTTCTG TGTGTGTTTG
TGGTGAGTGC CGCATGGTGG GGGCGTCTCG GCCTCTCTCC TCTCTCTCCA CTGTTTTCTC
TTTCTGTGTG TCTGTTTCCA TTCTATCTCC ACCTTCTTCC CTCCGTCTTT TGCTTTTCTA
TCTCCACTTC TCCACACCCC TCTCTCCCTG CGTCTCTGTG TCTCCCTCTT CCTCTGTCTT
GTTTTTTTCC CACCGTCTGC CTCTTCTGTT CCCTGTCACA TCCAACTTCC ACCGGTTTCT
CCAGCTCTCT CCTCAGTTCC TTCTCTCATG AGCACACCTG CCTCTGTGCT CGTATTCCTG
GACTCCTCTC TCTCCACTGT CATATCTTCT CATTCATTTT CCCAGTCTCT CTCTGTCTCT
TGCTCTCCCC CTCTCTGTCA CTCTGTCTCT GTCTCTCTCT TTCTCTCTCT CTCTCTGTGT
CTCTCTGTCT GGCTCTCTCT CTGTCTCTCT CTCCATCTCT CTCTCTCTCT CCCCCCCGTC
ACCCTGTCTC TGTCTCTCTC TGTCTGTGTG TCTCTCTGTC TTTCTCTCTC TCCATCTCTC
TCTGTCTCTC TCTCTCTCTC TCTCTCTCTC CCTCTCTCCC TCCTCCCGTG ACTCCCTCTC
TCAGTCCATC TCTTCCTCCC TCTCTCAGCC CCTTCGTGCC CTTTCCTCTG ACACTCCCCA
CCCTGGTTTC CTGACTCCAC CACTAGATCC ACCACCTCCA GCAACTGGGA ACCCTCCCCT
GCCCACCCTG CCCTGGGGTC CCCTCCCAGG ATTCCTTCTA GATTATAGCA TCTTCCCTGG
GCGGGTTCTC ATGAACAATT GTGGCTGCTT TTTTGGCCAG ACAGGGGAGG GAGGGGATGG
GATCAGGGAG TCCTGGAATG GGAACTAGGC AATAAAAAAA AAAAAATGTC AGAAGCAGGG
CGGCGGGAGG TGGGGGCAGG GCCAGCTGTC CTTACCAGGG ATAAAAGGCT TTGCCAGTGT
GACTAGGAAG AGAGACACCT CCCCTCCTTC CTTCATCAAG **ACATCAAGGA GGGACCTGTG**
CCCTGCTCCA CATCCTCCCA CCTGCCGCCC GCAGAGCCTG CAGGCCCCGC CCCCCTCGTC
TCTGGTCCCT ACCTCTCTGC TGTGTCTTCA TGTCCCTGAG GGTCTTGGGC TCTGGGTAAG
TGCCCCTTGC TGTCTCTGCC TCTCAGCCCC CGGTTCTGTT GAAGGTTCCT TCTCTCTCAC
TTTTTCTCTG CATTTGACAG **GACCTGGCCC TCAGCCCCTA AAATGTTCCT CCTGCTGACA**
GCACTTCAAG TCCTGGCTAT AGGTAAGAGA ACGGTTGGGT ATGACACAAG GGGGTCCCCT
GGAGACTCTG AGAAGAGATG GGGATGGGTC CTTGGGGCCC CTGGATGCTC ATGGTGACCT
CATAAGAAAG AGCAGGGAGT GGTTTGGGGG TCATGGTGGG GGAACGTGCT GGAGGCCTAA
ATTCCTAGTT GTGGAGGTGC TAGGGAATTG TGGGGCCGGG GAGAGAGGTG TTTATAAGGT
CTGGTGCAAA ATACATAAGG AATCTTAGGG AACTATTAGG TCCTGAGTGG GTCATAGCAG
AAAGATCACG GGGCTCTACC TGACTGTGTT AGGAAAGAAA CAATGTCAGA AAGATGTTTT
GTTGTCAGAG GGAAGGTGGA GAAGGATGAT GGGATGGCGG GATCGTGGCA TGGGGTGGCG
GGATCGTGGC ATGGGTGTGT GAGGTGGATG GGGGCAAGTG TGGGGCAAGA GATGGCGGAT
CCTTGGGGTC CCACTGAGTG GGAACGTTGG GGAGGAGACA GGGAGGTCCT TGAATGTGTT
GGGGAAGGAC TCATTGGGGG GAAATGTGGC ATATTTCGAG AAGTGATCAC AGAAATTATG
GGAGCATAGA GCTAAGGGTC GTAGATGTAG CAAGGCCCTG GATAAGGTGG CCACGGCACA
AAATAAGAGA TGCTACGGAG GTGACTTGGG AGGTGAGTCA GAAAGCTCTC CGTGCTGGGG

FIGURE 41 (CONT'D)

CAATAACGGG GTCAATATTG GGCATGTCTC ACCCTGGGTG GGACAGATAG AGGCGGGCAG
TTTAGGGGTT AGACCAAAAG GAAGGGGATT TGTCAGTTTT GGAATCCTAC AAACTTGTGG
AGTGGAGAGT GTTTGCTCAT CTACTTTCCC CACCCAATCC TGTCCACTCC TAG**CCATGAC**
ACAGAGCCAA GAGGATGAGA ACAAGATAAT TGGTGGCCAT ACGTGCACCC GGAGCTCCCA
GCCGTGGCAG GCGGCCCTGC TGGCGGGTCC CAGGCGCCGC TTCCTCTGCG GAGGCGCCCT
GCTTTCAGGC CAGTGGGTCA TCACTGCTGC TCACTGCGGC CGCCCGTAAG TGACCCCCTC
CCCTGTCCCT GTACCTAGTG AATTCCAGAG TCTAAAGCCC TAGAGCTGAG CTGAGAACCT
GGATCTCTGT ATAGAACCCA ATGTAGTGGC TGGCTCCTGG TTTGAGGTCT AGAGAAGAGC
CTGGAACAAA AACACAGCTC GGGATGTGGG CTCCTCCATA AATCTCGAAC TCAGCATAGG
TTCTGAAAGC AGATGGGCAG CTTGGAACCC ATGGACCTGC TGAGAACCGA ACATCTGATC
CAGTGATTCT TCCAGAGGCC ACACATTACA TCGAGACCAA GCTTAGCCCA TTCCAGATTG
GTGGCTGAAT TCAGGACCCC GTCTACATTC AGAAACTCAG GACACTACGT AGAACTCAGA
GCCCAGTTCA GGACCTGCAG TCTAGCCATA AATCCAGAAC TAGAACGCTG CTCACAGCTG
GAACATACAA CTCTAAGAAT AGAGGCAAAA CCTGGAGGCT GTTTCACACC CAAGGTTTAG
TTCAGAGTCT AGTCTATAGC TCCGCTATGA GCAGACTTCA ACCCAGTGTT TGAATCCCAG
AATGTGGCGG GTGCGGTGGC TCATGCCTAT AATCCTAGCA CTTTGGGATG CTGAGGCAGG
CAGATCACCT GAGGTCAGGA GTTCGAGACC AGCCTGAGCA ACATAGAGAA ACCCTGTCTC
TACTAAAAAT GCAAAATTAG CCAGGCATGG TGGCACATGC CTGTAATCCC AGCCACTCGG
GAGGCTGAGG CAGGAGAATC ACTTGAACCT GGGAGGCGGA GGTTGCAGTG AGTCAAGATC
GCACCATTGC ACTCCAGGCT AGGCAACAAG AGCGAAACTC CATATCAATC AATCAATCAA
TAAATCCCAG AATGCAGATC CTAATCAGAA GCCCCATATA AAACCTAGAC CCCTCCTAAA
TTCTAGATCT GAACTTACAA CCCAGACCCC AGCCAAGAGG TCAAAATGCC TATAAGCCAT
ATCTATGCCA TAAACAGGTC AGTCTAGAAC CTAGAGATCA AAGCTCAGGC CAGAGTCTAG
AATATAAAGG CCAGAATGCA AACCAGACTC TAGAATCTTG GATCCGGGCC ATAACCTAGA
GCTCCAACTA GAACCCAGAG CCCAACCTGA GGTCAAGGGC TAGGGCCAGA GTCCAGAACC
AAGAGCCCTA TAATCCAATA TGAAACAGAC CTGTAGAGGC TGGGTGCGGT GGCTCACGCC
TGTAATCCCA GCACTTTGGG AGGCTGAGGC GGGAGAATCA CTTGAACTGG GAGTTGGAGG
TCGAGAGTGA GCTGAGATCG TGCCACTGCA CTCCAGCCTA GGTGACAGAG CGAGACTCCA
TCACAAAAAA AAAATAAATA AATAAATCAA GTCATAATCC AGGTTCGATC TAGAATCCTG
ATCTTAGCAT AGAGTCAAAA GTTTAAGATG TCTAGAACTC AGAACCCAGG CTAGAAACAG
AATGGTGCCT ACTCCGGAAT ATCAGTTCCG ATTTAGAGCC TAGACTCATA ACGCAGTTTC
GCTTAGGACT CAATGCACCG AGCCCAGCAC AGACCCTGGC ACGGAGCCAA GCTCTCCCAA
TCATCACCTT CTTCCCAAGC CAGGAGCTGG AGCCCAGCCC AAGAGCGGAA GGAGAGGCAG
CTGGGGCTGG GCCGAGAGAA TGCCCTGGCC ATGGGGAAGG GCACAGGAGG CCAAGAATGC
TCGGCCTGCA GTTAGTGAGA AGCAGGCTAG ACCTCGGGGA AGACTCGTCA CCCGGCCAGG
GAACCGGGCT GGAGGGTGGG GAGGAGTCTC TGGCTCAGAC CCTGAGCAGC GCTTCTCTTG
GGGGTCGTGG CCAG**GATCCT TCAGGTTGCC CTGGGCAAGC ACAACCTGAG GAGGTGGGAG**
GCCACCCAGC AGGTGCTGCG CGTGGTTCGT CAGGTGACGC ACCCCAACTA CAACTCCCGG
ACCCACGACA ACGACCTCAT GCTGCTGCAG CTACAGCAGC CCGCACGGAT CGGGAGGGCA
GTCAGGCCCA TTGAGGTCAC CCAGGCCTGT GCCAGCCCCG GGACCTCCTG CCGAGTGTCA
GGCTGGGGAA CTATATCCAG CCCCATCGGT GAGGACTCCT GCGTCTTGGA AAGCAGGGGA
CTGGGCCTGG GCTCCTGGGT CTCCAGGAGG TGGAGCTGGG GGGACTGGGG CTCCTGGGTC
TGAGGGAGGA GGGGCTGGGC CTGGACTCCT GGGTCTGAGG GAGGAGGGGG CTGAGGCCTG
GACTCCTGGG TCTCAAGGAG GAGGAGCTGG GCCTGGACTC ATACGTCTGA GGGAGGAGGG
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GACTCCTGGG TCTGAGGGAG GAGGGGCTGG GGGCCTGGAC TCCTGGGTCT GAGGGAGGAG
GTGCTGGGGC TGGACTCCTG GGTCGGAAGG AGGAGGGGCT GGGGGCCTGG ACCCTTGGGT
CTTATGGGAG GGTAGACCCA GTTATAACCC TGCAGTGTCC CCCAG**CCAGG TACCCCGCCT**
CTCTGCAATG CGTGAACATC AACATCTCCC CGGATGAGGT GTGCCAGAAG GCCTATCCTA
GAACCATCAC GCCTGGCATG GTCTGTGCAG GAGTTCCCCA GGGCGGGAAG GACTCTTGTC
AGGTAAGGCC CAGGATGGGA GCTGTGGTAG GGATTATTTG GGACTGGGAT TTAAGCAAAT
GATGTCAGGA GCATGGAAGT CTGCAGAGGT CTTCAGAAGA GAGTGAACCG CAGGCACAGA
GAGATTCCGA TAGCCAGGCC ACCCTGCTTC CTAGCCCTGT GCCCCCTGGG TAATGGACTC
AGAGCATTCA TGCCTCAGTT TCCTCATCTG TCAGGTGGGA GTAACCCTCT TAGGGTAGTT
GGTGGAATGG GATGAGGCAG GTTGGGGAAA GATCGCAGAG TGGCCTCTGC TCATATGGGT

FIGURE 41 (CONT'D)

CTGGGAAAGG CTGTGCTGAG GCTTCTAGAA ATCTTAATGC ATCCTTGAGG GAGGCAGAGA
TGGGGAAATA GAAAAAGAGA GACACACAAA TGTTCTACAG TTGGAGCGAA CAGAGAGGGG
CCTGGTGAGA TTCAAGGGAC AGGCAGGTGC ACACAGAGAC AGAGCCAGAC CCAGCGGAGA
GGGAAGGAAG TGCCCCGACC TCCGGGGCTG AGACCTCAGA GCTGGGGCAG GACTGTGTCC
CTAACTGTCC ACCAGTGTCT CTGCCTGTCT CCCTGTGTCT GCTTCTCGGG TTCTCTGTGC
CATGGTGGCT CTGGCTACCT GTCCATCAGT GTCTCCATTT CTGTTCCTCC CCCTCAG**GGT**
GACTCTGGGG GACCCCTGGT GTGCAGAGGA CAGCTCCAGG GCCTCGTGTC TTGGGGAATG
GAGCGCTGCG CCCTGCCTGG CTACCCCGGT GTCTACACCA ACCTGTGCAA GTACAGAAGC
TGGATTGAGG AAACGATGCG GGACAAA*TGA* TGGTCTTCAC GGTGGGATGG ACCTCGTCAG
CTGCCCAGGC CCTCCTCTCT CTACTCAGGA CCCAGGAGTC CAGGCCCCAG CCCCTCCTCC
CTCAGACCCA GGAGTCCAGG CCCCCAGCCC CTCCTCCCTC AGACCCGGGA GTCCAGGCCC
CCAGCCCCTC CTCCCTCAGA CCCAGGAGTC CAGGCCCCAG CCCCTCCTCC CTCAGACCCG
GGAGTCCAGG CCCCCAGCCC CTCCTCCCTC AGACCCAGGA GTCCAGGCCC CAGTCCCTCC
TCCCTCAGAC CCAGGAGTCC AGGCCCCCAG CCCCTCCTCC CTCAGACCCA GGAATCCAGG
CCCAGCCCCT CCTCCCTCAG ACCCAGGAGC CCCAGTCCCC CAGCCCCTCC TCCTTGAGAC
CCAGGAGTCC AGGCCCAGCC CCTCCTCCCT CAGACCCAGG AGCCCCAGTC CCCAGCATCC
TGATCTTTAC TCCGGCTCTG ATCTCTCCTT TCCCAGAGCA GTTGCTTCAG GCGTTTTCTC
CCCACCAAGC CCCCACCCTT GCTGTGTCAC CATCACTACT CAAGACCGGA GGCACAGAGG
GCAGGAGCAC AGACCCCTTA AACCGGCATT GTATTCCAAA GACGACAATT TTTAACACGC
TTAGTGTCTC TAAAAACCG**A ATAAA**TAATG AC**AATAAA**AA TGGAATCATC CTAAATTGTA
TTCATTCATC CATGTGTTTA CTTTTTATTT TTTGAGACAA GGTCTTGCTC AGTCTCCTGG
TGAAATGCTG TAACGCAATC ATAGCTCACT GCAACCGTGA CCTCCTGGGC TCCAGTGATC
CTCTTACCTC AGCCTCCCGA GTAGCTGGGA CCACAGGTGC CCGTCACCAT GCCCCGCTAC
TTTTTAAATT TTGTGTAGAG ATGAGGTTTC CCTGTGTTGC TCAGGCTGGT CTCGAACACC
TGACCCCAAG CAATCCGCCT ACGTCGGTTT CCCAAAGTGC CGGGATTGCA GGCGTGAGCT
GCCGCGCCCA GCCTTATCCA TCCAATTAAT GACTTCAAGA AACATGTACA CAGTGGCCCC
ACCATGCCAA GCCAGGAGCT GTGTACTGAC AAGTGGCTGC CTCCCTCTTT GCGTGTTTTT
CCTTGGGAGT CCCCCGTCCA CCCCACTGTA TCAGGTTTCT AGACGGAAAC ACCTCAGCCC
TGCAGAGTGA CCTTGAGCAT GACTGCCTTC TACCAGCCTC CTCCCTGGAG CCCCTGTGGT
CCAGGGTAGG GAACTAAGTG CCTTGTTTCC TGGAAAATTC TATGCAAATG AAGATGTCCT
CATTTTCCTA ATCAGATCTC AGGTGAGGAG AGTTGAGTTA ATCACAGGCT TCAGTTCCTG
CCCAGGCAAA GCCCTTCTCT CATTTTATTA ATTTATTTCC ACTCTTCATC TCTGGCTCTG
CTCCCCTCCC TCCCCACAGG CACCGACATA AATGGCTTTG AGTGCCCTGC ATCCTTGGAA
AACAAGGCAG TGTCACAGTG TACTGTTTCT AATTTACATG AAACCATTGT GTTAGGAATC
TCATTCTCTT TCTTACTTTC ACTCATCAAC AGCTATTGAG CACCTACTAC GGGCCAGGCA
TTGGTCTATT TATTAGGCAC CTGCTATACA CCAGGCATTG TTCTGGGTGC TGGAGGAAGA
ACTGTGAGCA AGCCAGTCAG AATCCCTGCC CTCACAGAAC TTATATTCTA GCAGGAGATG
ACAGACAAGA AGCCATAAAC ATAATTTTAA AATAAAGCAG AGTCCCTATG AGTAACGAGG
TCAATAAACT TGGGCTGGGC GGCAGGCCCA ATGTGTGCCA GGGCCAGCTC ATACATGCTC
GCAAGAGTCT ACCAGCAAAT TTTCAGGAAT TTCGAGAACC AGTTGCTAAA TGCAGCCATC
ATTAAAAATT AAATTACATA AGCGTATAAT TACATAATTG ATTAAAAAAA TTGTCAGTAA
ATACTCAAAA CTCAACTGTT GCTAATTATT TCAACTAATA CCTATGCTTG GGAGTGAGAT
ATGTCTCTTG TACTACGTCT GTAATGATGA GTTTCTGCAC ACCTCTTTCC AACTCCCCAA
CTCTGTCTGC ACCAGTAGCT TGACAATAGC CAAAGAAGAA GTATTTACTG CACTGAAATT
GAAAAACACT ATAGATAGGG CTTTGCCGGA CAGTCATTGC TAAACCTTTA CCAGGCACCC
TTGGATGGGT CTGCCTGGGA ATGACCTCAT GATCTTAGTG TCTGTCTTCT CAAAGTTCTG
TGCTTGGATA CTGCAGAGTA TAGCTAAAAT AGAATGTTGT ACTCACCTTA TGTTCTATGG
GGACAGCACA GTATTGGGGA ACCCTAAGGT GGCAGGTCTG GGACATGCAC GAAAGATTGC
TGGGAAGTAG AGGCTCCCTC CTTTTCCTCA TCCTCCCACC CCATCCTCCA GTGTCTGGTA
ACCACCATTC TACTCTCTGC TTCTAAGAGT CTGAGTTTTT TAGATTTCAC ATGTAAGTGA
GATCATGCAG TAATTGTCAT TCTGTGTCTG ACCTATTTCA CTTAACACAG TGTCCTCCCG
GTCCATCCAT GTTGTCACAA ATGACAGGAT TTCTTTCTTT TATAAGGCAG AATAATATTA
AATTATACTG ATACTAATAT ATTACATTTC CTTTATCCAT TCATCCATCA ACAGACACAT

FIGURE 42

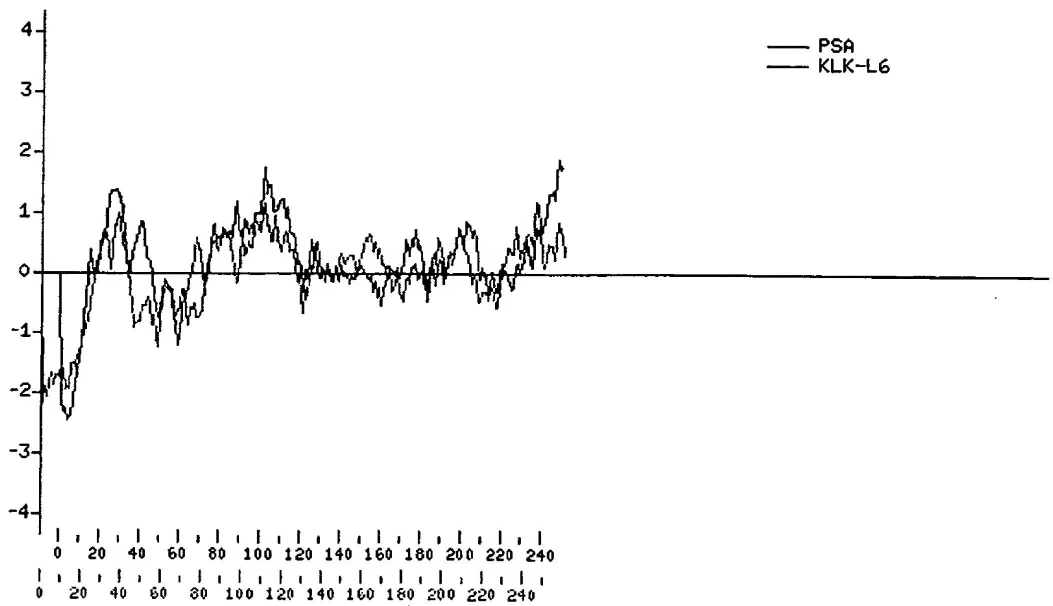
PSA
KLK-L6
4
3
2
1
0
-1
-2
-3
-4
0 20 40 60 80 100 120 140 160 180 200 220 240
0 20 40 60 80 100 120 140 160 180 200 220 240

FIGURE 43

```
                1             15 16            30 31            45 46            60 61            75 76            90
 1 PSA          --------------- ---MWVPVVFLTLSV TWIGAAPLILS---- ------RIVGGWECE KHSQPWQVLVASRG- -RAVCGGVLVHPQWV   60
 2 hK2          --------------- ---MWDLVLSIALSV GCTGAVPLIQS---- ------RIVGGWECE KHSQPWQVAVYSHG- -WAHCGGVLVHPQWV   60
 3 hK1          --------------- ---MWFLVLCLALSL GGTGAAPPIQS---- ------RIVGGWECE QHSQPWQAALYHFS- -TFQCGGILVHRQWV   60
 4 HSCCE        --------------M ARSLLLPLQILLLSL ALETAGEEAQGD--- ------KIIDGAPCA RGSHPWQVALLSGN- -QLHCGGVLVNERWV   65
 5 zyme         --------------- --MKKLMVVLSLIAA AWAEEQ--------- -----NKLVHGGPCD KTSHPYQAALYTSG- -HLLCGGVLIHPLWV   57
 6 KLK-L6       --------------- --MFLLLTALQVLAI AMTQSQEDE------ -----NKIIGGHTCT RSSQPWQAALLAGPR RRFLCGGALLSGQWV   62
 7 TLSP         --------------- --MRILQLILLALAT GLVGGETR------- -------IIKGFECK PHSQPWQAALFEKT- -RLLCGATLIAPRWL   57
 8 KLK-L4       --------------- --MWPLALVIASLTL ALSGGVSQESSKVLN TNGTSGFLPGGYTCF PHSQPWQAALLVQG- -RLLCGGVLVHPKWV   71
 9 NES1         MRAPHLHLSAASGAR ALAKLLPLLMAQLWA AEAALLPQNDTR-L- -----DPEAYGAPCA RGSQPWQVSLFNGL- -SFHCAGVLVDQSWV   81
10 KLK-L5       --------------- -MGLSIFLLLCVLGL SQAATPKIFN----- ----------GTECG RNSQPWQVGLFEGT- -SLRCGGVLIDHRWV   57
11 neuropsin    ----------MGRPR PRAAKTWMFLLLLGG AWAGHSRAQE----- -----DKVLGGHECQ PHSQPWQAALFQGQ- -QLLCGGVLVGGNWV   68
12 prostase     ---------MATAGN PWGWFLGYLILGVAG SLVSGSCS------- ------QIINGEDCS PHSQPWQAALVMEN- -ELFCSGVLVHPQWV   66

                91           105 106          120 121          135 136          150 151          165 166          180
 1 PSA          LTAAHCIRNKSVILL GRHSLFHP-EDTGQV FQVSHSFPHPLYDMS LLKNRFLRPGDDSSH DLMLLRLSEPAE-LT DAVKVMDLPTQ-EPA  147
 2 hK2          LTAAHCLKKNSQVWL GRHNLFEP-EDTGQR VPVSHSFPHPLYNMS LLKHQSLRPDEDSSH DLMLLRLSEPAK-IT DVVKVLGLPTQ-EPA  147
 3 hK1          LTAAHCISDNYQLWL GRHNLFDD-ENTAQF VHVSESFPHPGFNMS LLENHTRQADEDYSH DLMLLRLTEPADTIT DAVKVVELPTE-EPE  148
 4 HSCCE        LTAAHCKMNEYTVHL GSDTLGD---RRAQR IKASKSFRHPGY--S T---------QTHVN DLMLVKLNSQAR-LS SMVKKVRLPSRCEP-  139
 5 zyme         LTAAHCKKPNLQVFL GKHNLRQR-ESSQEQ SSVVRAVIHPDYD-- ---------AASHDQ DIMLLRLARPAK-LS ELIQPLPLERD-CSA  133
 6 KLK-L6       ITAAHCGRPILQVAL GKHNLRRW-EATQQV LRVVRQVTHPNYN-- ---------SRTHDN DLMLLQLQQPAR-IG RAVRPIEVTQA-CAS  138
 7 TLSP         LTAAHCLKPRYIVHL GQHNLQKE-EGCEQT RTATESFPHPGFNNS L-------PNKDHRN DIMLVKMASPVS-IT WAVRPLTLSSR-CVT  137
 8 KLK-L4       LTAAHCLKEGLKVYL GKHALGRV-EAGEQV REVVHSIPHPEYRRS P-------THLNHDH DIMLLELQSPVQ-LT GYIQTLPLSHNNRLT  152
 9 NES1         LTAAHCGNKPLWARV GDDHLLLL-QG-EQL RRTTRSVVHPKYHQG SGP---ILPRRTDEH DLMLLKLARPVV-PG PRVRALQLPYR-CAQ  164
10 KLK-L5       LTAAHCSGSRYWVRL GEHSLSQL-DWTEQI RHSGFSVTHPGYLGA S---------TSHEH DLRLLRLRLPVR-VT SSVQPLPLPND-CAT  135
11 neuropsin    LTAAHCKKPKYTVRL GDHSLQNK-DGPEQE IPVVQSIPHPCYNSS D--------VEDHNH DLMLLQLRDQAS-LG SKVKPISLADH-CTQ  147
12 prostase     LSAAHCFQNSYTIGL GLHSLEADQEPGSQM VEASLSVRHPEYN-- ---------RPLLAN DLMLIKLDESVS-ES DTIRSISIASQ-CPT  143
```

FIGURE 43(CONT'D)

```
               181.          195 196          210 211          225 226          240 241          255 256          270
 1 PSA         LGTTCYASGWGSIEP EEFLTPKKLQCVDLH VISNDVCAQVHPQKV TKFMLCAGRWTGGKS TCSGDSGGPLVCNGV LQGITSWG-SEPCAL   236
 2 hK2         LGTTCYASGWGSIEP EEFLRPRSLQCVSLH LLSNDMCARAYSEKV TEFMLCAGLWTGGKD TCGGDSGGPLVCNGV LQGITSWG-PEPCAL   236
 3 hK1         VGSTCLASGWGSIEP ENFSFPDDLQCVDLK ILPNDECKKAHVQKV TDFMLCVGHLEGGKD TCVGDSGGPLMCDGV LQGVTSWG-YVPCGT   237
 4 HSCCE       PGTTCTVSGWGTTTS PDVTFPSDLMCVDVK LISPQDCTKVYKDLL ENSMLCAGIPDSKKN ACNGDSGGPLVCRGT LQGLVSWG-TFPCGQ   228
 5 zyme        NTTSCHILGWGKTAD --GDFPDTIQCAYIH LVSREECEHAYPGQI TQNMLCAGDEKYGKD SCQGDSGGPLVCGDH LRGLVSWG-NIPCGS   220
 6 KLK-L6      PGTSCRVSGWGTISS PIARYPASLQCVNIN ISPDEVCQKAYPRTI TPGMVCAGVPQGGKD SCQGDSGGPLVCRGQ LQGLVSWG-MERCAL   227
 7 TLSP        AGTSCLISGWGSTSS PQLRLPHTLRCANIT IIEHQKCENAYPGNI TDTMVCASVQEGGKD SCQGDSGGPLVCNQS LQGIISWG-QDPCAI   226
 8 KLK-L4      PGTTCRVSGWGTTTS PQVNYPKTLQCANIQ LRSDEECRQVYPGKI TDNMLCAGTKEGGKD SCEGDSGGPLVCNRT LYGIVSWG-DFPCGQ   241
 9 NES1        PGDQCQVAGWGTTAA RRVKYNKGLTCSSIT ILSPKECEVFYPGVV TNNMICAGLDR-GQD PCQSDSGGPLVCDET LQGILSWG-VYPCGS   252
10 KLK-L5      AGTECHVSGWGITNH PRNPFPDLLQCLNLS IVSHATCHGVYPGRI TSNMVCAGGVP-GQD ACQGDSGGPLVCGGV LQGLVSWGSVGPCGQ   224
11 neuropsin   PGQKCTVSGWGTVTS PRENFPDTLNCAEVK IFPQKKCEDAYPGQI TDGMVCAGSSK-GAD TCQGDSGGPLVCDGA LQGITSWG-SDPCGR   235
12 prostase    AGNSCLVSGWGLLAN --GRMPTVLQCVNVS VVSEEVCSKLYDPLY HPSMFCAGGGHDQKD SCNGDSGGPLICNGY LQGLVSFG-KAPCGQ   230

               271          285 286          300 301          315 316          330 331          345 346          360
 1 PSA         PERPSLYTKVVHYRK WIKDTIVANP----- ------     261
 2 hK2         PEKPAVYTKVVHYRK WIKDTIAANP----- ------     261
 3 hK1         PNKPSVAVRVLSYVK WIEDTIAENS----- ------     262
 4 HSCCE       PNDPGVYTQVCKFTK WINDTMKKHR----- ------     253
 5 zyme        KEKPGVYTNVCRYTN WIQKTIQAK------ ------     244
 6 KLK-L6      PGYPGVYTNLCKYRS WIEETMRDK------ ------     251
 7 TLSP        TRKPGVYTKVCKYVD WIQETMKNN------ ------     250
 8 KLK-L4      PDRPGVYTRVSRYVL WIRETIRKYETQQQK WLKGPQ     277
 9 NES1        AQHPAVYTQICKYMS WINKVIRSN------ ------     276
10 KLK-L5      DGIPGVYTYICKYVD WIRMIMRNN------ ------     248
11 neuropsin   SDKPGVYTNICRYLD WIKKIIGSKG----- ------     260
12 prostase    VGVPGVYTNLCKFTE WIEKTVQAS------ ------     254
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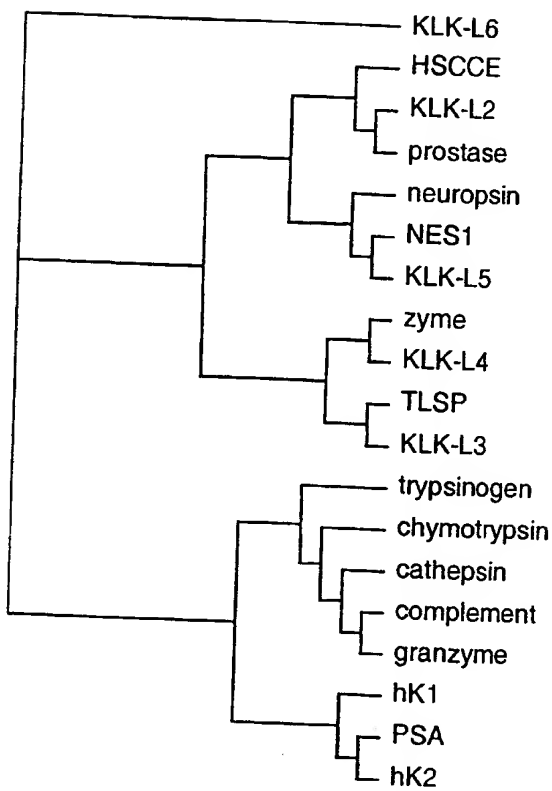
FIGURE 44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trypsinogen
chymotrypsin
cathepsin
complement
granzyme
hK1
PSA
hK2

Sequence Listing

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KLK-L1 na

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301 tcagcccagc agtggaatcc aggagcccag aggtggccgg gtaagaggcc tggtggtccc
361 ccactaaaag cctgcagtgt tcatgatcca actctcccta cagctccatg tcgctggatt
421 ctcagcctct gtgccttctg tctccacatc tctctagaca gatctctcac tgtctctagt
481 taggagtcac tgtctctagt taggggtctc tctgtctctc tgaatctata tctccatgtc
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1021 tgtctgtccg tgcttggggg cacagtgttt gttaatgttc aggtgtgact cagtgtcctc
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1201 gttgtacctg tgagactctg acagtttgtg cctctgaata tctggtggag tgacaacagt
1261 gtaatgatga tatggggaca ggggaagccg agggtgcagg agattgtgct tcctggggcg
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Figure 4 Sequence

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SEQ.ID. NO. 5

Table 8 Sequence

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SEQ.ID. NO. 6

Table 8 Sequence

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SEQ.ID. NO. 7

Table 8 Sequence

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SEQ.ID. NO. 8

Table 8 Sequence

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SEQ.ID. NO. 9

Table 8 Sequence

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SEQ.ID. NO. 10

Table 8 Sequence

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SEQ.ID. NO. 11

Table 8 Sequence

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Table 8 Sequence

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SEQ.ID. NO. 15

Table 11

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SEQ.ID. NO. 16

Table 11

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SEQ.ID. NO. 17

Table 11

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SEQ.ID. NO. 18

Table 11

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SEQ.ID. NO. 19

Table 11

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SEQ.ID. NO. 20

Table 11

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SEQ.ID. NO. 21

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SEQ. ID. NO. 23

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SEQ. ID. NO. 24

Table 13

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SEQ. ID. NO. 25

Table 13

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SEQ. ID. NO. 26

Table 13

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SEQ. ID. NO. 27

Table 14

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SEQ. ID. NO. 28

TABLE 14

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SEQ. ID. NO. 29

TABLE 14

AACATCAGCATCCTGGAGAA

SEQ. ID. NO. 30

TABLE 14

CTTCGGCCTCTCTTGGTCTT

SEQ. ID. NO. 31

TABLE 14

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SEQ. ID. NO. 32

TABLE 14

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SEQ. ID. NO. 33

TABLE 14

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SEQ. ID. NO. 34

TABLE 14

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TABLE 14

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TABLE 14

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TABLE 14

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TABLE 14

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SEQ. ID. NO. 40

TABLE 14

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TABLE 14

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TABLE 14

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SEQ. ID. NO. 43

KLK-L4 NA

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Table 15

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Table 15

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Table 15

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Table 15

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SEQ.ID.NO. 50

Table 15

ATATTGCCTAGGTGGATGTG

SEQ.ID.NO. 51

Table 15

AAGACTTCAAGGAGCCAAGC

SEQ.ID.NO. 52

Table 15

GACCCTTCACCTCCCAAAAT

SEQ.ID.NO. 53

Table 15

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SEQ.ID.NO. 54

Table 15

GGTGATCTGCGCCCTGGTCCT

SEQ.ID.NO. 55

Table 15

AGGTGTCCGGTGGAGGTGGCA

SEQ.ID.NO. 56

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11761 cagattcaga gactcagggc agggaaagga aggctgcaga cagacagaca gacagaggga

SEQ.ID.NO. 57

KLK-L5 AA

LSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLIDHRWVLTAAHCSG
SRYWVRLGEHSLSQLDWTEQIRHSGFSVTHPGYLGASTSHEHDLRLLRLRLP
VRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPRNPFPDLLQCLNLSIVSHA
TCHGVYPGRITSNMVCAGGVPGQDACQ

SEQ.ID.NO. 58

KLK-L5 AA – alternatively spliced

MGLSIFLLLCVLGLSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID
HRWVLTAAHCSGRPIPGSAPVPQPLHRLPCHLPWCVSRENHEQHGVCRRRPG
AGCLPG

SEQ.ID.NO. 59

KLK-L5 AA – alternatively spliced

MGLSIFLLLCVLGLSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID
HRWVLTAAHCSGSRYWVRLGEHSLSQLDWTEQIRHSGFSVTHPGYLGASTS
HEHDLRLLRLRLPVRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPRNPFPD
LLQCLNLSIVSHATCHGVYPGRITSNMVCAGGVPGQDACQGDSGGPLVCGG
VLQGLVSWGSVGPCGQDGIPGVYTYICNSTLVGLGTSWNFNSCQPF

SEQ.ID.NO. 60

KLK-L5-AA

MGLSIFLLLCVLGLSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID
HRWVLTAAHCSGSRYWVRLGEHSLSQLDWTEQIRHSGFSVTHPGYLGASTS
HEHDLRLLRLRLPVRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPRNPFPD
LLQCLNLSIVSHATCHGVYPGRITSNMVCAGGVPGQDACQGDSGGPLVCGG
VLQGLVSWGSVGPCGQDGIPGVYTYICKYVDWIRMIMRNN

SEQ.ID.NO. 61

Table 17

TCAGCCAGGCAGCCACACCG

SEQ.ID.NO. 62

Table 17

TTGGTGATGCCCCAGCCTGA

SEQ.ID.NO. 63

Table 17

CCACACCGAAGATTTTCAAT

SEQ.ID.NO. 64

Table 17

GCCCCTCCTTCATTTATA

SEQ.ID.NO. 65

KLK-L6 NA

1 atcgtgtaat caccgccaca tccagtgcaa agctgattcg tcaccacaga gcagctccct
61 cctgccaccc catccctggg tcccaagaga accctttctt aaaagaggga gttcttgacg
121 ggtgtggtgg ctcatgcctg taatccttgc actttgggag gccaaggagg gtggatcatt
181 tgaggtcagg agtttgagac cagactggcc aacatggtga aaccctgtct ttactaaaaa
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361 ctgcactcca gccggggcta aagagtgaga ctctgtctca aaaaaaaaaa aaagaaaaag
421 aaaaaaagaa aaaaaaataa aataaataaa taaataaaat aaatttaaaa atttaaaaat
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3001 attcctagtt gtggaggtgc tagggaattg tggggccggg gagagaggtg tttataaggt
3061 ctggtgcaaa atacataagg aatcttaggg aactattagg tcctgagtgg gtcatagcag
3121 aaagatcacg gggctctacc tgactgtgtt aggaaagaaa caatgtcaga aagatgtttt
3181 gttgtcagag ggaaggtgga gaaggatgat gggatggcgg gatcgtggca tggggtggcg
3241 ggatcgtggc atgggtgtgt gaggtggatg ggggcaagtg tggggcaaga gatggcggat
3301 ccttggggtc ccactgagtg ggaacgttgg ggaggagaca gggaggtcct tgaatgtgtt
3361 ggggaaggac tcattggggg gaaatgtggc atatttcgag aagtgatcac agaaattatg
3421 ggagcataga gctaagggtc gtagatgtag caaggccctg gataaggtgg ccacggcaca
3481 aaataagaga tgctacggag gtgacttggg aggtgagtca gaaagctctc cgtgctgggg

3541 caataacggg gtcaatattg ggcatgtctc accctgggtg ggacagatag aggcgggcag
3601 tttaggggtt agaccaaaag gaaggggatt tgtcagtttt ggaatcctac aaacttgtgg
3661 agtggagagt gtttgctcat ctactttccc cacccaatcc tgtccactcc tagccatgac
3721 acagagccaa gaggatgaga acaagataat tggtggccat acgtgcaccc ggagctccca
3781 gccgtggcag gcggccctgc tggcgggtcc caggcgccgc ttcctctgcg gaggcgccct
3841 gctttcaggc cagtgggtca tcactgctgc tcactgcggc cgcccgtaag tgaccccctc
3901 ccctgtccct gtacctagtg aattccagag tctaaagccc tagagctgag ctgagaacct
3961 ggatctctgt atagaaccca atgtagtggc tggctcctgg tttgaggtct agagaagagc
4021 ctggaacaaa aacacagctc gggatgtggg ctcctccata aatctcgaac tcagcatagg
4081 ttctgaaagc agatgggcag cttggaaccc atggacctgc tgagaaccga acatctgatc
4141 cagtgattct tccagaggcc acacattaca tcgagaccaa gcttagccca ttccagattg
4201 gtggctgaat tcaggacccc gtctacattc agaaactcag gacactacgt agaactcaga
4261 gcccagttca ggacctgcag tctagccata aatccagaac tagaacgctg ctcacagctg
4321 gaacatacaa ctctaagaat agaggcaaaa cctggaggct gtttcacacc caaggtttag
4381 ttcagagtct agtctatagc tccgctatga gcagacttca acccagtgtt tgaatcccag
4441 aatgtggcgg gtgcggtggc tcatgcctat aatcctagca ctttgggatg ctgaggcagg
4501 cagatcacct gaggtcagga gttcgagacc agcctgagca acatagagaa accctgtctc
4561 tactaaaaat gcaaaattag ccaggcatgg tggcacatgc ctgtaatccc agccactcgg
4621 gaggctgagg caggagaatc acttgaacct gggaggcgga ggttgcagtg agtcaagatc
4681 gcaccattgc actccaggct aggcaacaag agcgaaactc catatcaatc aatcaatcaa
4741 taaatcccag aatgcagatc ctaatcagaa gccccatata aaacctagac ccctcctaaa
4801 ttctagatct gaacttacaa cccagacccc agccaagagg tcaaaatgcc tataagccat
4861 atctatgcca taaacaggtc agtctagaac ctagagatca aagctcaggc cagagtctag
4921 aatataaagg ccagaatgca aaccagactc tagaatcttg gatccgggcc ataacctaga
4981 gctccaacta gaacccagag cccaacctga ggtcaagggc tagggccaga gtccagaacc
5041 aagagcccta taatccaata tgaaacagac ctgtagaggc tgggtgcggt ggctcacgcc
5101 tgtaatccca gcactttggg aggctgaggc gggagaatca cttgaactgg gagttggagg
5161 tcgagagtga gctgagatcg tgccactgca ctccagccta ggtgacagag cgagactcca
5221 tcacaaaaaa aaaataaata aataaatcaa gtcataatcc aggttcgatc tagaatcctg
5281 atcttagcat agagtcaaaa gtttaagatg tctagaactc agaacccagg ctagaaacag

5341 aatggtgcct actccggaat atcagttccg atttagagcc tagactcata acgcagtttc
5401 gcttaggact caatgcaccg agcccagcac agaccctggc acggagccaa gctctcccaa
5461 tcatcacctt cttcccaagc caggagctgg agcccagccc aagagcggaa ggagaggcag
5521 ctggggctgg gccgagagaa tgccctggcc atggggaagg gcacaggagg ccaagaatgc
5581 tcggcctgca gttagtgaga agcaggctag acctcgggga agactcgtca cccggccagg
5641 gaaccgggct ggagggtggg gaggagtctc tggctcagac cctgagcagc gcttctcttg
5701 ggggtcgtgg ccaggatcct tcaggttgcc ctgggcaagc acaacctgag gaggtgggag
5761 gccacccagc aggtgctgcg cgtggttcgt caggtgacgc accccaacta caactcccgg
5821 acccacgaca acgacctcat gctgctgcag ctacagcagc ccgcacggat cgggagggca
5881 gtcaggccca ttgaggtcac ccaggcctgt gccagccccg ggacctcctg ccgagtgtca
5941 ggctggggaa ctatatccag ccccatcggt gaggactcct gcgtcttgga aagcagggga
6001 ctgggcctgg gctcctgggt ctccaggagg tggagctggg gggactgggg ctcctgggtc
6061 tgagggagga ggggctgggc ctggactcct gggtctgagg gaggaggggg ctgaggcctg
6121 gactcctggg tctcaaggag gaggagctgg gcctggactc atacgtctga gggaggaggg
6181 gctggagcct ggactcctgg gtctcaagga ggaggggctg ggcctggact tctgggtctg
6241 agggaggagg ggctggggac ctggactccc gggtctgagg gaggagggac tgggggtctg
6301 gactcctggg tctgagggag gaggggctgg gggcctggac tcctgggtct gagggaggag
6361 gtgctggggc tggactcctg ggtcggaagg aggaggggct gggggcctgg acccttgggt
6421 cttatgggag ggtagaccca gttataaccc tgcagtgtcc cccagccagg taccccgcct
6481 ctctgcaatg cgtgaacatc aacatctccc cggatgaggt gtgccagaag gcctatccta
6541 gaaccatcac gcctggcatg gtctgtgcag gagttcccca gggcgggaag gactcttgtc
6601 aggtaaggcc caggatggga gctgtggtag ggattatttg ggactgggat ttaagcaaat
6661 gatgtcagga gcatggaagt ctgcagaggt cttcagaaga gagtgaaccg caggcacaga
6721 gagattccga tagccaggcc accctgcttc ctagccctgt gccccctggg taatggactc
6781 agagcattca tgcctcagtt tcctcatctg tcaggtggga gtaaccctct tagggtagtt
6841 ggtggaatgg gatgaggcag gttggggaaa gatcgcagag tggcctctgc tcatatgggt
6901 ctgggaaagg ctgtgctgag gcttctagaa atcttaatgc atccttgagg gaggcagaga
6961 tggggaaata gaaaaagaga gacacacaaa tgttctacag ttggagcgaa cagagagggg
7021 cctggtgaga ttcaagggac aggcaggtgc acacagagac agagccagac ccagcggaga
7081 gggaaggaag tgccccgacc tccggggctg agacctcaga gctggggcag gactgtgtcc

7141 ctaactgtcc accagtgtct ctgcctgtct ccctgtgtct gcttctcggg ttctctgtgc
7201 catggtggct ctggctacct gtccatcagt gtctccattt ctgttcctcc ccctcagggt
7261 gactctgggg gacccctggt gtgcagagga cagctccagg gcctcgtgtc ttggggaatg
7321 gagcgctgcg ccctgcctgg ctaccccggt gtctacacca acctgtgcaa gtacagaagc
7381 tggattgagg aaacgatgcg ggacaaatga tggtcttcac ggtgggatgg acctcgtcag
7441 ctgcccaggc cctcctctct ctactcagga cccaggagtc caggccccag cccctcctcc
7501 ctcagaccca ggagtccagg cccccagccc ctcctccctc agacccggga gtccaggccc
7561 ccagcccctc ctccctcaga cccaggagtc caggccccag cccctcctcc ctcagacccg
7621 ggagtccagg cccccagccc ctcctccctc agacccagga gtccaggccc cagtccctcc
7681 tccctcagac ccaggagtcc aggcccccag cccctcctcc ctcagaccca ggaatccagg
7741 cccagcccct cctccctcag acccaggagc cccagtcccc cagcccctcc tccttgagac
7801 ccaggagtcc aggcccagcc cctcctccct cagacccagg agccccagtc cccagcatcc
7861 tgatctttac tccggctctg atctctcctt tcccagagca gttgcttcag gcgttttctc
7921 cccaccaagc ccccacccctt gctgtgtcac catcactact caagaccgga ggcacagagg
7981 gcaggagcac agacccctta aaccggcatt gtattccaaa gacgacaatt tttaacacgc
8041 ttagtgtctc taaaaaccga ataaataatg acaataaaaa tggaatcatc ctaaattgta
8101 ttcattcatc catgtgttta ctttttattt tttgagacaa ggtcttgctc agtctcctgg
8161 tgaaatgctg taacgcaatc atagctcact gcaaccgtga cctcctgggc tccagtgatc
8221 ctcttacctc agcctcccga gtagctggga ccacaggtgc ccgtcaccat gccccgctac

SEQ.ID.NO. 66

KLK-L6 AA

MTQSQEDENKIIGGHTCTRSSQPWQAALLAGPRRRFLCGGALLSGQWVITAA
HCGRPILQVALGKHNLRRWEATQQVLRVVRQVTHPNYNSRTHDNDLMLLQL
QQPARIGRAVRPIEVTQACASPGTSCRVSGWGTISSPIARYPASLQCVNINISPD
EVCQKAYPRTITPGMVCAGVPQGGKDSCQGDSGGPLVCRGQLQGLVSWGM
ERCALPGYPGVYTNLCKYRSWIEETMRDK

SEQ.ID.NO. 67

KLK-L6 AA

MFLLLTALQVLAIAMTQSQEDENKIIGGHTCTRSSQPWQAALLA
GPRRRFLCGGALLSGQWVITAAHCGRPILQVALGKHNLRRWEATQQVLRVV
RQVTHPNYNSRTHDNDLMLLQLQQPARIGRAVRPIEVTQACASPGTSCRVSG
WGTISSPIARYPASLQCVNINISPDEVCQKAYPRTITPGMVCAGVPQGGKDSC

QGDSGGPLVCRGQLQGLVSWGMERCALPGYPGVYTNLCKYRSWIEETMRD
K

SEQ.ID.NO. 68

Figure 9

prostase

MATAGNPWGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVME
NELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSQMVEASLSV
RHPEYNRPLLANDLMLIKLDESVSESDTIRSISIASQCPTAGNSCLVSGWGLLA
NGRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSG
GPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEK

SEQ.ID.NO. 69

Figure 9

EMSP

MATAGNPWGWFLGYLILGVAGSLVSGEMSPSCSQIINGEDCSPHSQPWQAAL
VMENELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSQMVEAS
LSVRHPEYNRPLLANDLMLIKLDESVSESDTIRSISIASQCPTAGNSCLVSGWG
LLANGRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNG
DSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEK 249

SEQ.ID.NO. 70

Figure 9

KLK-L2

MATARPPWMWVLCALITALLLGVTEHVLANNDVSCDHPSNTVPSGSNQDLG
AGAGEDARSDDSSSRIINGSDCDMHTQPWQAALLLRPNQLYCGAVLVHPQW
LLTAAHCRKKVFRVRLGHYSLSPVYESGQQMFQGVKSIPHPGYSHPGHSNDL
MLIKLNRRIRPTKDVRPINVSSHCPSAGTKCLVSGWGTTKSPQVHFPKVLQCL
NISVLSQKRCEDAYPRQIDDTMFCAGDKAGRDSCQGDSGGPVVCNGSLQGL
VSWGDYPCARPNRPGVYTNLCKFTKWIQE

SEQ.ID.NO. 71

Figure 9

zyme

MKKLMVVLSLIAAAWAEEQNKLVHGGPCDKTSHPYQAALYTSGHLLCGGV

LIHPLWVLTAAHCKKPNLQVFLGKHNLRQRESSQEQSSVVRAVIHPDYDAAS
HDQDIMLLRLARPAKLSELIQPLPLERDCSANTTSCHILGWGKTADGDFPDTI
QCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCGDH
LRGLVSWGNIPCGSKEKPGVYTNVCRYTNWIQK

SEQ.ID.NO. 72

Figure 9

neuropsin

MGRPRPRAAKTWMFLLLLGGAWAGHSRAQEDKVLGGHECQPHSQPWQAA
LFQGQQLLCGGVLVGGNWVLTAAHCKKPKYTVRLGDHSLQNKDGPEQEIPV
VQSIPHPCYNSSDVEDHNHDLMLLQLRDQASLGSKVKPISLADHCTQPGQKC
TVSGWGTVTSPRENFPDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG
ADTCQGDSGGPLVCDGALQGITSWGSDPCGRSDKPGVYTNICRYLDWIKKTL
SPMRILQLILLALATGLVG

SEQ.ID.NO. 73

Figure 9

TLSP

GETRIIKGFECKPHSQPWQAALFEKTRLLCGATLIAPRWLLTAAHCLKPRYIV
HLGQHNLQKEEGCEQTRTATESFPHPGFNNSLPNKDHRNDIMLVKMASPVSI
TWAVRPLTLSSRCVTAGTSCLISGWGSTSSPQLRLPHTLRCANITIIEHQKCEN
AYPGNITDTMVCASVQEGGKDSCQGDSGGPLVCNQSLQGIISWGQDPCAITR
KPGVYTKVCKYVDWIQE

SEQ.ID.NO. 74

Figure 9

PSA

MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG
GVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDM
SLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYAS
GWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGK
STCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKD

SEQ.ID.NO. 75

Figure 9

KLK2

MWDLVLSIALSVGCTGAVPLIQSRIVGGWECEKHSQPWQVAVYSHGWAHCG
GVLVHPQWVLTAAHCLKKNSQVWLGRHNLFEPEDTGQRVPVSHSFPHPLYN
MSLLKHQSLRPDEDSSHDLMLLRLSEPAKITDVVKVLGLPTQEPALGTTCYAS
GWGSIEPEEFLRPRSLQCVSLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKD
TCGGDSGGPLVCNGVLQGITSWGPEPCALPEKPAVYTKVVHYRKWIKD

SEQ.ID.NO. 76

Figure 9

KLK1

MWFLVLCLALSLGGTGAAPPIQSRIVGGWECEQHSQPWQAALYHFSTFQCGG
ILVHRQWVLTAAHCISDNYQLWLGRHNLFDDENTAQFVHVSESFPHPGFNMS
LLENHTRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEEPEVGSTCLASG
WGSIEPENFSFPDDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDT
CVGDSGGPLMCDGVLQGVTSWGYVPCGTPNKPSVAVRVLSYVKWIED

SEQ.ID.NO. 77

Figure 9

trypsinogen

MNPLLILTFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGSLI
NEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNN
DIMLIKLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQC
LDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQ
GVVSWG-DGCAQKNKPGVYTKVYNYVKWIKN

SEQ.ID.NO. 78

Figure 17, 27, 36, 43

PSA

MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG
GVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDM
SLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYAS
GWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGK
STCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVA
NP

SEQ.ID.NO. 79

Figure 17, 27, 36, 43

KLK2

MWDLVLSIALSVGCTGAVPLIQSRIVGGWECEKHSQPWQVAVYSHGWAHCG
GVLVHPQWVLTAAHCLKKNSQVWLGRHNLFEPEDTGQRVPVSHSFPHPLYN
MSLLKHQSLRPDEDSSHDLMLLRLSEPAKITDVVKVLGLPTQEPALGTTCYAS
GWGSIEPEEFLRPRSLQCVSLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKD
TCGGDSGGPLVCNGVLQGITSWGPEPCALPEKPAVYTKVVHYRKWIKDTIAA
NP

SEQ.ID.NO. 80

Figure 17, 27, 36, 43

KLK1

MWFLVLCLALSLGGTGAAPPIQSRIVGGWECEQHSQPWQAALYHFSTFQCGG
ILVHRQWVLTAAHCISDNYQLWLGRHNLFDDENTAQFVHVSESFPHPGFNMS
LLENHTRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEEPEVGSTCLASG
WGSIEPENFSFPDDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDT
CVGDSGGPLMCDGVLQGVTSWGYVPCGTPNKPSVAVRVLSYVKWIEDTIAE
NS

SEQ.ID.NO. 81

Figure 17, 27, 36, 43

prostase

MATAGNPWGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVME
NELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSQMVEASLSV
RHPEYNRPLLANDLMLIKLDESVS-
ESDTIRSISIASQCPTAGNSCLVSGWGLLANG—
RMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGP
LICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEKTVQAS

SEQ.ID.NO. 82

Figure 17, 27

trypsinogen

MNPLLILTFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGSLI
NEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNN
DIMLIKLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQC
LDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQ
GVVSWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANS

SEQ.ID.NO. 83

Figure 17, 27, 36, 43

neuropsin

MGRPRPRAAKTWMFLLLLGGAWAGHSRAQEDKVLGGHECQPHSQPWQAA
LFQGQQLLCGGVLVGGNWVLTAAHCKKPKYTVRLGDHSLQNKDGPEQEIPV
VQSIPHPCYNSSDVEDHNHDLMLLQLRDQASLGSKVKPISLADHCTQPGQKC
TVSGWGTVTSPRENFPDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG
ADTCQGDSGGPLVCDGALQGITSWGSDPCGRSDKPGVYTNICRYLDWIKKIIG
SKG

SEQ.ID.NO. 84

Figure 17, 27, 36, 43

zyme

MKKLMVVLSLIAAAWAEEQNKLVHGGPCDKTSHPYQAALYTSGHLLCGGV
LIHPLWVLTAAHCKKPNLQVFLGKHNLRQRESSQEQSSVVRAVIHPDYDAAS
HDQDIMLLRLARPAKLSELIQPLPLERDCSANTTSCHILGWGKTADGDFPDTI
QCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCGDH
LRGLVSWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK

SEQ.ID.NO. 85

Figure 27

EMSP

MATAGNPWGWFLGYLILGVAGSLVSGEMSPSCSQIINGEDCSPHSQPWQAAL
VMENELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSQMVEAS
LSVRHPEYNRPLLANDLMLIKLDESVSESDTIRSISIASQCPTAGNSCLVSGWG
LLANGRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNG
DSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEKTVQAS

SEQ.ID.NO. 86

Figure 27

TLSP

MRI-LQLILLALATGLVGGETRIIKGFECKPHSQPWQAALFEKTRLLC
GATLIAPRWLLTAAHCLKPRYIVHLGQHNLQKEEGCEQTRTATESFPHPGFNN
SLPNKDHRNDIMLVKMASPVSITWAVRPLTLSSRCVTAGTSCLISGWGSTSSP
QLRLPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGP
LVCNQSLQGIISWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN

SEQ.ID.NO. 87

Figure 27

HSCEE

MARSLLLPLQILLLSLALETAGEEAQGDKIIDGAPCARGSHPWQVALLSGNQL
HCHSCCEGGVLVNERWVLTAAHCKMNEYTVHLGSDTLGDRRAQRIKASKSF
RHPGYSTQTHVNDLMLVKLNSQARLSSMVKKVRLPSRCEPPGTTCTVSGWG
TTTSPDVTFPDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNG
DSGGPLVCRGTLQGLVS WGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR

SEQ.ID.NO. 88

Figure 27

NES1

MRAPHLHLSAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDPEAYGAP
CARG SQPWQVSLFNGLSFH CAGVLVDQSWVLTAAHCGNKPLWARVGDDH
LL-LLQG-EQLRRTT RSVVHPKYHQGSGPI LPRRTDEHDLML LKLARPVV-
PGPRVR ALQLPYR-CAQPGDQ CQVAGWGTTAARRVK YNKGLTCSSITILSP
KECEVFYPGVVTNNM ICAGLDR-GQDPCQS DSGGPLVCDETLQGI LSWG-
VYPCGSAQHPAVYTQICKYMSWINK VIRSN